

TOWNSEND and TOWNSEND and CREW LLP

Two Embarcadero Center, 8th Floor

San Francisco, CA 94111-3834

(415) 326-2400

Atty. Docket No. 15389-002600US"Express Mail" Label No. EM197111574USDate of Deposit August 14, 1997

## PATENT APPLICATION

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Sir:

Transmitted herewith for filing is the

☒ patent application of☐ design patent application of☐ continuation-in-part patent application of

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By 

Inventor(s): Thomas R. Cech, Joachim Lingner, Toru Nakamura, Karen B. Chapman, Gregg B. Morin, Calvin B. Harley and William Andrews.

For: HUMAN TELOMERASE CATALYTIC SUBUNIT: DIAGNOSTIC AND THERAPEUTIC METHODS

☒ This application claims priority from each of the following Application Nos./filing dates:08/854,050/May 9, 1997; 08/851,843/May 6, 1997; 08/846,017/April 25, 1997; 08/844,419/April 18, 1997;08/724,643/October 1, 1996.

☐ Please amend this application by adding the following before the first sentence: --This application claims the benefit of U.S. Provisional Application No. 60/\_\_\_\_\_, filed \_\_\_\_\_, the disclosure of which is incorporated by reference.--

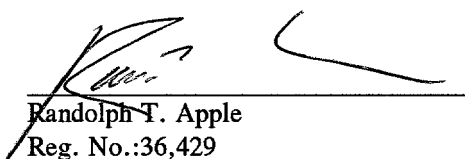
Enclosed are:

☒ 201 pages of specification, 7 pages of claims, 0 page of abstract and 44 sheets of informal drawings.☒ A ☐ signed ☒ unsigned Declaration & Power of Attorney.☒ Return postcard

In view of the Unsigned Declaration as filed with this application and pursuant to 37 CFR §1.53(d), Applicant requests deferral of the filing fee until submission of the Missing Parts of Application.

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Telephone:  
(415) 326-2400

  
Randolph T. Apple

Reg. No.:36,429

Attorneys for Applicant

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## HUMAN TELOMERASE CATALYTIC SUBUNIT: DIAGNOSTIC AND THERAPEUTIC METHODS

The present application is a Continuation-In-Part application of U.S. Patent Appln. Ser. No. 08/854,050, filed May 9, 1997 which is Continuation-In-Part of U.S. Patent Appln. Ser. No. 08/851,843, filed May 6, 1997 which is a Continuation-In-Part application of U.S. Patent Appln. Ser. No. 08/846,017, filed April 25, 1997 which is a Continuation-in-Part of U.S. Patent Appln. Ser. No. 08/844,419, filed April 18, 1997 which is a Continuation-in-Part of U.S. Patent Appln. Ser. No. 08/724,643, filed on October 1, 1996. Each of the aforementioned applications is explicitly incorporated herein by reference in its entirety and for all purposes.

The present application further incorporates each of co-pending U.S. Patent Applications Serial No. \_\_\_\_\_ (Attorney Docket No. 015389-002500, entitled "TELOMERASE REVERSE TRANSCRIPTASE", filed August 14, 1997), in its entirety and for all purposes.

This invention was made with Government support under Grant No. GM28039, awarded by the National Institute of Health. The Government has certain rights in this invention.

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### FIELD OF THE INVENTION

The present invention is related to novel nucleic acids and polypeptides encoding the catalytic subunit of telomerase. In particular, the present invention is directed to the catalytic subunit of telomerases from *Euplotes aediculatus*, *Schizosaccharomyces pombe*, *Tetrahymena thermophila*, and humans. The invention provides methods and compositions relating to medicine, molecular biology, chemistry, pharmacology, and medical diagnostic and prognostic technology.

## BACKGROUND OF THE INVENTION

The following discussion is intended to introduce the field of the present invention to the reader. The citation of various references in this section is not to be construed as an admission of prior invention.

5           It has long been recognized that complete replication of the ends of eukaryotic chromosomes requires specialized cell components (Watson, 1972, *Nature New Biol.*, 239:197; Olovnikov, 1973, *J. Theor. Biol.*, 41:181). Replication of a linear DNA strand by conventional DNA polymerases requires an RNA primer, and can proceed only 5' to 3'. When the RNA bound at the extreme 5' ends of eukaryotic  
10       chromosomal DNA strands is removed, a gap is introduced, leading to a progressive shortening of daughter strands with each round of replication. This shortening of *telomeres*, the protein-DNA structures physically located on the ends of chromosomes, is thought to account for the phenomenon of cellular senescence (cell aging) of normal human somatic cells *in vitro* (see, e.g., Goldstein, 1990, *Science* 249:1129) and *in vivo*  
15       (see, e.g., Martin et al., 1979, *Lab. Invest.* 23:86; Goldstein et al., 1969, *Proc. Natl. Acad. Sci. USA* 64:155; and Schneider and Mitsui, 1976, *Proc. Natl. Acad. Sci. USA*, 73:3584).

          The length and integrity of telomeres is thus related to entry of a cell into a senescent stage (i.e., loss of proliferative capacity). Moreover, the ability of a  
20       cell to maintain (or increase) telomere length may allow a cell to escape senescence, i.e., to become immortal.

          The structure of telomeres and telomeric DNA has been investigated in numerous systems (see, e.g., Harley and Villeponteau, 1995, *Curr. Opin. Genet. Dev.* 5:249). In most organisms, telomeric DNA consists of a tandem array of very simple  
25       sequences; in humans and other vertebrates telomeric DNA consists of hundreds to thousands of tandem repeats of the sequence TTAGGG. Methods for determining and modulating telomere length in cells are described in PCT Publications WO 95/13382 and WO 96/41016.

          The maintenance of telomeres is a function of a telomere-specific DNA  
30       polymerase known as *telomerase*. Telomerase is a ribonucleoprotein (RNP) that uses a

portion of its RNA moiety as a template for telomere repeat DNA synthesis (Morin, 1997, *Eur. J. Cancer* 33:750; Yu et al., 1990, *Nature* 344:126; Singer and Gottschling, 1994, *Science* 266:404; Autexier and Greider, 1994, *Genes Develop.*, 8:563; Gilley et al., 1995, *Genes Develop.*, 9:2214; McEachern and Blackburn, 1995, *Nature* 367:403; 5 Blackburn, 1992, *Ann. Rev. Biochem.*, 61:113; Greider, 1996, *Ann. Rev. Biochem.*, 65:337). The RNA components of human and other telomerases have been cloned and characterized (see, PCT Publication WO 96/01835 and Feng et al., 1995, *Science* 269:1236). However, the characterization of the protein components of telomerase has been difficult. In part, this is because it has proved difficult to purify the telomerase 10 RNP, which is present in extremely low levels in cells in which it is expressed. For example, it has been estimated that human cells known to express high levels of telomerase activity may have only about one hundred molecules of the enzyme per cell.

Consistent with the relationship of telomeres and telomerase with the proliferative capacity of a cell (i.e., the ability of the cell to divide indefinitely), 15 telomerase activity is detected in immortal cell lines and an extraordinarily diverse set of tumor tissues, but is not detected (i.e., was absent or below the assay threshold) in normal somatic cell cultures or normal tissues adjacent to a tumor (see, U.S. Patent Nos. 5,629,154; 5,489,508; 5,648,215; and 5,639,613; see also, Morin, 1989, *Cell* 59: 521; Shay and Bacchetti 1997, *Eur. J. Cancer* 33:787; Kim et al., 1994, *Science* 20 266:2011; Counter et al., 1992, *EMBO J.* 11:1921; Counter et al., 1994, *Proc. Natl. Acad. Sci. U.S.A.* 91, 2900; Counter et al., 1994, *J. Virol.* 68:3410). Moreover, a correlation between the level of telomerase activity in a tumor and the likely clinical outcome of the patient has been reported (e.g., U.S. Patent No. 5,639,613, *supra*; Langford et al., 1997, *Hum. Pathol.* 28:416). Telomerase activity has also been 25 detected in human germ cells, proliferating stem or progenitor cells, and activated lymphocytes. In somatic stem or progenitor cells, and in activated lymphocytes, telomerase activity is typically either very low or only transiently expressed (see, Chiu et al., 1996, *Stem Cells* 14:239; Bodnar et al., 1996, *Exp. Cell Res.* 228:58; Taylor et al., 1996, *J. Invest. Dermatology* 106: 759).

30 Human telomerase is an ideal target for diagnosing and treating human



diseases relating to cellular proliferation and senescence, such as cancer. Methods for diagnosing and treating cancer and other telomerase-related diseases in humans are described in U.S. Patent Nos. 5,489,508, 5,639,613, and 5,645,986. Methods for predicting tumor progression by monitoring telomerase are described in U.S. Patent No. 5,639,613. The discovery and characterization of the catalytic protein subunit of human telomerase would provide additional useful assays for telomerase and for disease diagnosis and therapy. Moreover, cloning and determination of the primary sequence of the catalytic protein subunit would allow more effective therapies for human cancers and other diseases related to cell proliferative capacity and senescence.

### BRIEF SUMMARY OF THE INVENTION

The present invention provides an isolated, substantially pure, or recombinant protein preparation of a telomerase reverse transcriptase protein. In one embodiment the protein has an amino acid sequence:

Trp-R<sub>1</sub>-X<sub>7</sub>-R<sub>1</sub>-R<sub>1</sub>-R<sub>2</sub>-X-Phe-Phe-Tyr-X-Thr-Glu-X<sub>8,9</sub>-R<sub>3</sub>-R<sub>3</sub>-Arg-R<sub>4</sub>-X<sub>2</sub>-Trp  
where X is any amino acid and a subscript refers to the number of consecutive residues, R<sub>1</sub> is leucine or isoleucine, R<sub>2</sub> is glutamine or arginine, R<sub>3</sub> is phenylalanine or tyrosine, and R<sub>4</sub> is lysine or histidine. In one embodiment the protein has a sequence of human TRT. In other embodiment, the invention relates to peptides and polypeptides sharing substantial sequence identity with a subsequence of such proteins.

In a related embodiment the invention provides an isolated, substantially pure or recombinant nucleic acid that encodes a telomerase reverse transcriptase protein. In one embodiment the protein has an amino acid sequence:

Trp-R<sub>1</sub>-X<sub>7</sub>-R<sub>1</sub>-R<sub>1</sub>-R<sub>2</sub>-X-Phe-Phe-Tyr-X-Thr-Glu-X<sub>8,9</sub>-R<sub>3</sub>-R<sub>3</sub>-Arg-R<sub>4</sub>-X<sub>2</sub>-Trp. In one embodiment the nucleic acid has a sequence of human TRT. In other embodiment, the invention relates to oligonucleotides and polynucleotides sharing substantial sequence identity with a subsequence of such nucleic acids.

In one aspect the invention provides isolated human telomerase comprising human telomerase reverse transcriptase (hTRT). In one embodiment the hTRT is associated with human telomerase RNA (hTR).

In one aspect the invention provides a method of detecting a human telomerase reverse transcriptase (hTERT) gene product in a biological sample by contacting the biological sample with a probe that specifically binds the gene product, wherein the probe and the gene product form a complex, and detecting the complex  
5 where the presence of the complex is correlated with the presence of the hTERT gene product in the biological sample. The gene product may be RNA, DNA or a polypeptide. Examples of probes in that may be used for detection include, but are not limited to, nucleic acids and antibodies.

In one embodiment the gene product is a nucleic acid which is detected  
10 by amplifying the gene and detecting detecting the amplification product, where the presence of the complex or amplification product is correlated with the presence of the hTERT gene product in the biological sample.

In one embodiment the biological sample is from a patient, such as a human patient. In another embodiment the biological sample includes at least one cell  
15 from an *in vitro* cell culture, such as a human cell culture.

The invention further provides a method of detecting the presence of at least one immortal or telomerase positive human cell in a biological sample comprising human cells by obtaining the biological sample comprising human cells; and detecting the presence in the sample of a cell having a high level of an hTERT gene product, where  
20 the presence of a cell having a high level of the hTERT gene product is correlated with the presence of immortal or telomerase positive cells in the biological sample.

The invention also provides a method for diagnosing a telomerase-related condition in a patient by obtaining a cell or tissue sample from the patient, determining the amount of a human telomerase reverse transcriptase (hTERT) gene  
25 product in the cell or tissue; and comparing the amount of hTERT gene product in the cell or tissue with the amount in a healthy cell or tissue of the same type, where a different amount of hTERT gene product in the sample from the patient and the healthy cell or tissue is diagnostic of a telomerase-related condition. In one embodiment the telomerase-related condition is cancer.

30 The invention further provides a method of diagnosing cancer in a

patient by obtaining a biological sample from the patient, and detecting a human telomerase reverse transcriptase (hTERT) gene product in the patient sample, where the detection of the hTERT gene product in the sample is correlated with a diagnosis of cancer.

5                   The invention further provides a method of diagnosing cancer in a patient by obtaining a patient sample, determining the amount human telomerase reverse transcriptase (hTERT) gene product in the patient sample; and comparing the amount of hTERT gene product with a normal or control value, where an amount of the hTERT gene product in the patient that is greater than the normal or control value is  
10       diagnostic of cancer.

                  The invention still further provides a method of diagnosing cancer in a patient, by obtaining a patient sample containing at least one cell; determining the amount of an hTERT gene product in a cell in the sample; and comparing the amount of hTERT gene product in the cell with a normal value for the cell, wherein an amount of  
15       the hTERT gene product greater than the normal value is diagnostic of cancer. In one embodiment the sample is believed to contain at least one malignant cell.

                  The invention still further provides a method of providing a prognosis for a cancer patient by determining the amount of hTERT gene product in a cancer cell obtained from the patient; and comparing the amount of hTERT in the cancer cell with a  
20       prognostic value of hTERT per cancer cell consistent with a prognosis for the cancer; where an amount of hTERT per cell in the sample that is at the prognostic value provides the particular prognosis.

                  The invention still further provides a method for monitoring the ability of an anticancer treatment to reduce the proliferative capacity of cancer cells in a  
25       patient, by making a first measurement of the amount of an hTERT gene product in at least one cancer cell from the patient; making a second measurement of the level of the hTERT gene product in at least one cancer cell from the patient, wherein the anticancer treatment is administered to the patient before or at the same time as the second measurement; and comparing the first and second measurements, where a lower level  
30       of the hTERT gene product in the second measurement is correlated with the ability of an

anticancer treatment to reduce the proliferative capacity of cancer cells in the patient.

The invention also provides kits for the detection of a hTRT gene or gene product. In one embodiment the kit includes a container including a molecule selected from an hTRT nucleic acid or subsequence thereof, an hTRT polypeptide or  
5 subsequence thereof, and an anti-hTRT antibody.

The invention also provides methods of treating human diseases. In one aspect the invention provides a method for increasing the proliferative capacity of a vertebrate cell, such as a mammalian cell, by introducing a recombinant polynucleotide into the cell, wherein said polynucleotide comprises a sequence encoding a human  
10 telomerase reverse transcriptase (hTRT) polypeptide. In one embodiment the hTRT polypeptide has a sequence of SEQ. ID. NO. 2. In one embodiment the sequence is operably linked to a promoter. In one embodiment the hTRT has telomerase catalytic activity. In one embodiment the cell is human, such as a cell in a human patient. In an alternative embodiment, the cell is cultured *in vitro*. In a related embodiment the cell is  
15 introduced into a human patient.

The invention further provides a method for treating a human disease by introducing recombinant hTRT polynucleotide into at least one cell in a patient. In one embodiment a gene therapy vector is used. In a related embodiment, the method further consists of introducing into the cell a polynucleotide comprising a sequence encoding  
20 human telomerase RNA, for example an hTR polynucleotide operably linked to a promoter.

The invention also provides a method for increasing the proliferative capacity of a vertebrate cell, said method comprising introducing into the cell an effective amount of a human telomerase reverse transcriptase (hTRT) polypeptide. In  
25 one embodiment the the hTRT polypeptide has telomerase catalytic activity. The invention further provides cells and cell progeny with increased proliferative capacity.

The invention also provides pharmacological compositions containing a pharmaceutically acceptable carrier and a molecule selected from: an hTRT polypeptide, a polynucleotide encoding a hTRT polypeptide, and an hTRT nucleic acid  
30 or subsequence thereof.

The invention also provides a method for treatment of a condition associated with an elevated level of telomerase activity within a cell, comprising introducing into said cell a therapeutically effective amount of an inhibitor of said telomerase activity, wherein said inhibitor is an hTRT polypeptide or a hTRT polynucleotide. In one embodiment the inhibitor is a polypeptide comprising the sequence of SEQ. ID. NO: 2 or 4, or a subsequence thereof. In additional embodiments the polypeptide inhibits a TRT activity, such as binding of endogenous TRT to telomerase RNA.

The invention also provides a vaccine comprising an hTRT polypeptide and an adjuvant.

## DESCRIPTION OF THE FIGURES

**Figure 1** shows highly conserved residues in TRT motifs 0, 1, 2, and 3. Identical amino acids are indicated with an asterisk (\*), while the similar amino acid residues are indicated by a circle (●).

**Figure 2** shows the location of telomerase-specific and RT sequence motifs of telomerase proteins. Locations of telomerase-specific motif T and conserved RT motifs 1, 2 and A-E are indicated by colored boxes. Bottom, the open rectangle labeled HIV-1 RT delineates the portion of this protein shown in Figure 3. Colored residues are highly conserved in all RTs and shown as space-filled residues in Figure 3.

**Figure 3** shows the crystal structure of the p66 subunit of HIV-1 reverse transcriptase (Brookhaven code 1HNV). Color-coding of RT motifs matches that in Figure 2. The view is from the back of the right hand to enable all motifs to be seen.

**Figure 4** shows multiple sequence alignment of telomerase RTs and members of other RT families (Sc\_al, cytochrome oxidase group II intron 1-encoded protein from *S. cerevisiae* mitochondria, Bm\_R1, reverse transcriptase from *Bombyx mori* R1 non-LTR retrotransposable element; HIV-1, human immunodeficiency virus reverse transcriptase). TRT con and RT con, consensus sequences for telomerase RTs and non-telomerase RTs. Amino acids are designated h, hydrophobic; p, polar; c, charged. Triangles show residues that are conserved among telomerase proteins but

different in other RTs. Rectangle below motif E highlights the primer grip region.

**Figure 5** shows expression of hTERT in telomerase-negative mortal cell strains and telomerase-positive immortal cell lines.

**Figure 6** shows a possible phylogenetic tree of telomerases and retroelements rooted with RNA-dependent RNA polymerases.

**Figure 7** shows a restriction map of lambda clone Gφ5.

**Figure 8** shows a map of chromosome 5p with the location of the hTERT gene indicated.

**Figure 9** shows the construction of a hTERT promoter-reporter plasmid.

**Figure 10** shows coexpression *in vitro* of the hTERT and hTR to produce catalytically active human telomerase.

**Figure 11** shows an alignment of four TRT proteins. "TRT con" shows a TRT consensus sequence. "RT con" shows consensus residues for other reverse transcriptases. Consensus residues in upper case indicate absolute conservation in TRT pr In the reverse transcriptase consensus, "h" indicates hydrophobic residues and "p" indicates polar residues.

**Figure 12** shows a Topoisomerase II cleavage site and NFκB binding site motifs in an hTERT intron (SEQ ID NO. 7).

**Figure 13** shows the sequence of the DNA encoding the *Euplotes* 123 kDa telomerase protein subunit.

**Figure 14** shows the amino acid sequence of the *Euplotes* 123 kDa telomerase protein subunit.

**Figure 15** shows the DNA and amino acid sequences of the *S. pombe* telomerase catalytic subunit.

**Figure 16** shows the hTERT cDNA sequence (Seq. ID. NO: 1)

**Figure 17** shows the hTERT protein (SEQ ID NO. 2 ) encoded by SEQ ID NO. 1.

**Figure 18** shows the sequence of clone 712562 (SEQ ID NO. 3 ).

**Figure 19** shows a 259 residue protein (SEQ ID NO. 10) encoded by SEQ ID NO. 3.

**Figure 20** shows the sequence of a nucleic acid encoding a  $\Delta 182$  variant polypeptide ( **SEQ ID NO: 4**).

**Figure 21** shows the sequence from an hTRT genomic clone (**SEQ ID NO. 6**).

5           **Figure 22** shows the effect of mutation of the TRT gene in yeast.

**Figure 23** shows the sequence of EST AA281296 (**SEQ ID NO. 8**).

**Figure 24** shows the sequence of the 182 basepairs (**SEQ ID NO. 9**) deleted in clone 712562.

10           **Figure 25** shows telomerase activity from BJ cells transfected with hTRT.

## DETAILED DESCRIPTION OF THE INVENTION

### I. INTRODUCTION

Telomerase is a ribonucleoprotein complex (RNP) comprising an RNA component and a catalytic protein component. The present invention relates to the cloning and characterization of the catalytic protein component of telomerase, hereinafter referred to as "TRT" (telomerase reverse transcriptase). TRT is so named because this protein acts as an RNA-dependent DNA polymerase (reverse transcriptase), using the telomerase RNA component (hereinafter, "TR") to direct synthesis of telomere DNA repeat sequences. Moreover, TRT is evolutionarily related to other reverse transcriptases (see **Example 12**).

In one aspect, the present invention provides TRT genes and proteins from ciliates, fungi, and vertebrates, especially mammals. In one important aspect, the present invention relates to the cloning and characterization of the catalytic protein component of human telomerase, hereinafter referred to as "hTRT." Human TRT is of extraordinary interest and value because, as noted *supra*, telomerase activity in human (and other mammalian cells) correlates with cell proliferative capacity, cell immortality, and the development of a neoplastic phenotype. For example, telomerase activity, and, as demonstrated in **Example 2**, *infra*, levels of human TRT gene products and are elevated in immortal human cells (such as malignant tumor cells and immortal cell lines) relative to mortal cells (such as most human somatic cells).

The present invention further provides methods and compositions valuable for diagnosis, prognosis, and treatment of human diseases and disease conditions, as described in some detail *infra*. Also provided are methods and reagents useful for immortalizing cells (*in vivo* and *ex vivo*), producing transgenic animals with desirable characteristics, and numerous other uses, many of which are described *infra*.

As described in detail in the above-referenced priority documents, TRT was initially characterized following purification of telomerase from the ciliate *Euplotes aediculatus*. Extensive purification of *E. aediculatus* telomerase, using RNA-affinity chromatography and other methods, yielded the protein "p123". Surprisingly, p123 is unrelated to proteins previously believed to constitute the protein subunits of



the telomerase holoenzyme (i.e., the p80 and p95 proteins of *Tetrahymena thermophila*). Analysis of the p123 DNA and protein sequences (Genbank Accession No. U95964; **Figures 13 and 14**) revealed reverse transcriptase (RT) motifs consistent with the role of p123 as the catalytic subunit of telomerase (see, e.g., **Figure 1**).

- 5 Moreover, p123 is related to a *S. cerevisiae* (yeast) protein, Est2p, which was known to play a role in maintenance of telomeres in *S. cerevisiae* (Genbank Accession No. S5396), but prior to the present invention was not recognized as encoding a telomerase catalytic subunit protein (see, e.g., Lendvay et al., 1996, *Genetics*, 144:1399).

- 10 In one aspect, the present invention provides reagents and methods for identifying and cloning novel TRTs using: nucleic acid probes and primers generated or derived from the TRT polynucleotides disclosed herein and in the above-referenced priority documents (e.g., for cloning TRT genes and cDNAs); antibodies that specifically recognize the motifs or motif sequences or other TRT epitopes (e.g., for expression cloning TRT genes or purification of TRT proteins); by screening computer  
15 databases; or other means. For example, as described in **Example 1**, PCR (polymerase chain reaction) amplification of *S. pombe* DNA was carried out with degenerate-sequence primers designed from the *Euplotes* p123 RT motifs B' and C. Of four prominent products generated, one encoded a peptide sequence homologous to *Euplotes* p123 and *S. cerevisiae* Est2p. Using this PCR product as a probe, the complete  
20 sequence of the *S. pombe* TRT homologue was obtained by screening of *S. pombe* cDNA and genomic libraries and amplifying *S. pombe* RNA by reverse transcription and PCR (RT-PCR). The complete sequence of the *S. pombe* gene ("trt1"; GenBank Accession No. AF015783; **Figure 15**) revealed that homology with p123 and Est2p was especially high in the reverse transcriptase motifs.

- 25 Amplification using degenerate primers derived from the telomerase RT motifs was also used to obtain TRT gene sequences in *Oxytricha trifallax* and *Tetrahymena thermophila*, as described in **Example 1**.

- The *Euplotes* p123, *S. pombe* trt1, and *S. cerevisiae* Est2p sequences of the invention were used in a search of a computerized database of human expressed  
30 sequence tags (ESTs) using the program BLAST (Altschul et al, 1990, *J. Mol. Biol.*

215:403). Searching this database with the Est2p sequence did not indicate a match, but searching with p123 and trt1 sequences identified a human EST (Genbank accession no. AA281296), as described in **Example 1**, putatively encoding a homologous protein. Complete sequencing of the cDNA clone containing the EST (hereinafter, "clone 712562"; see **SEQ. ID. NO: 3**) showed that seven RT motifs were present. However, this clone could not encode a contiguous human TRT because motifs B', C, D, and E were contained in a different open reading frame (ORF) than the more NH<sub>2</sub>-terminal motifs. In addition, the distance between motifs A and B' was substantially shorter than that of the three previously characterized TRTs. (Clone 712562 was obtained from the I.M.A.G.E. Consortium; Lennon et al., 1996, *Genomics* 33:151).

A cDNA clone, pGRN121, encoding a functional hTRT (**SEQ. ID. NO: 1**) was isolated from a cDNA library derived from the human 293 cell line as described in **Example 1**. Comparing clone 712562 with pGRN121 showed that clone 712562 has a 182 base pair (**SEQ ID NO: 9**) deletion between motifs A and B'. The additional 182 base pairs present in pGRN121 places all of the TRT motifs in a single open reading frame, and increases the spacing between the motif A and motif B' regions to a distance consistent with the other known TRTs. As is described *infra* in the Examples (e.g., **Example 7**), **SEQ. ID. NO: 1** encodes a catalytically active telomerase protein having the sequence of **SEQ ID NO: 2**. The polypeptide of **SEQ ID NO: 2** has 1132 residues and a calculated molecular weight of about 127 kilodaltons (kD).

As is discussed *infra*, and described in **Example 9**, *infra*, TRT cDNAs possessing the 182 basepair deletion characteristic of the clone 712562 are detected following reverse transcription of RNA from telomerase-positive cells (e.g., testis and 293 cells). hTRT RNAs lacking this 182 base pair sequence are referred to generally as "Δ182 variants" and may represent one, two, or several species. Although the hTRT variants lacking the 182 basepair sequence found in the pGRN121 cDNA (**SEQ ID NO. 1**) are unlikely to encode a fully active telomerase catalytic enzyme, they may play a role in telomerase regulation, as discussed *infra*, and/or have partial telomerase activity, such as telomere binding or hTR binding activity, as discussed *infra*.

Thus, in one aspect, the present invention provides an isolated polynucleotide with a sequence of a naturally occurring human TRT gene or mRNA including, but not limited to, a polynucleotide having the sequence of **SEQ ID NO: 1**. In a related aspect, the invention provides a polynucleotide encoding an hTRT protein, fragment, variant or derivative. In another related aspect, the invention provides sense and antisense nucleic acids that bind to an hTRT gene or mRNA. The invention further provides hTRT proteins, whether synthesized or purified from natural sources, antibodies and other agents that specifically bind an hTRT protein or a fragment thereof. The present invention also provides many novel methods, including methods that employ the aforementioned compositions, for example, by providing diagnostic and prognostic assays for human diseases, methods for developing therapeutics and methods of therapy, identification of telomerase-associated proteins, and methods for screening for agents capable of activating or inhibiting telomerase activity. Numerous other aspects and embodiments of the invention are provided *infra*.

The description below is organized by topic. Part II further describes amino acid motifs characteristic of TRT proteins. Parts III-VI describe, *inter alia*, nucleic acids, proteins, antibodies and purified compositions of the invention with particular focus on human TRT related compositions. Part VII describes, *inter alia*, methods and compositions of the invention useful for treatment of human disease. Part VIII describes production and identification of immortalized human cell lines. Part IX describes, *inter alia*, uses of the nucleic acids, polynucleotides, and other compositions of the invention for diagnosis of human diseases. Part X is a glossary of terms used in Parts I-IX. Part XI describes examples relating to specific embodiments of the invention. The organization of the description of the invention by topic and subtopic is to provide clarity, and not to be limiting in any way.

## II. TRT GENES AND PROTEINS

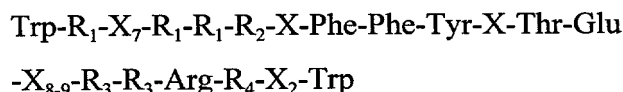
The present invention provides isolated and/or recombinant genes and proteins having a sequence of a telomerase catalytic subunit protein (i.e., telomerase reverse transcriptase), including, but not limited to, the naturally occurring forms of

such genes and proteins in isolated or recombinant form. Typically, TRTs are large, basic, proteins having *reverse transcriptase* (RT) and *telomerase-specific* amino acid motifs, as disclosed herein and in the above-referenced priority documents. Because these motifs are conserved across diverse organisms, TRT genes of numerous organisms may be obtained using the methods of the invention or identified using primers, nucleic acid probes, and antibodies of the invention, such as those specific for one or more of the motif sequences.

The seven RT motifs found in TRTs, while similar to those found in other reverse transcriptases, have particular hallmarks. For example, as shown in **Figure 4**, within the TRT RT motifs there are a number of amino acid substitutions (marked with arrows) in residues highly conserved among the other RTs. For example, in motif C the two aspartic acid residues (DD) that coordinate active site metal ions (see, Kohlstaedt et al., 1992, *Science* 256:1783; Jacobo-Molina et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90:6320; Patel et al., 1995, *Biochemistry* 34:5351) occur in the context hxDD(F/Y) in the telomerase RTs compared to (F/Y)xDDh in the other RTs (where h is a hydrophobic amino acid, and "x" is any amino acid; see Xiong et al., 1990, *EMBO J.* 9:3353; Eickbush, in *The Evolutionary Biology of Viruses*, (S. Morse, Ed., Raven Press, NY, p. 121, 1994)). Another systematic change characteristic of the telomerase subgroup occurs in motif E, where WxGxSx is a consensus sequence or is conserved among the telomerase proteins, whereas hLGxxh is characteristic of other RTs (Xiong et al., *supra*; Eickbush *supra*). This motif E is called the "primer grip", and mutations in this region have been reported to affect RNA priming but not DNA priming (Powell et al., 1997, *J. Biol. Chem.* 272:13262). Because telomerase requires a DNA primer, (e.g., the chromosome 3' end) it is not unexpected that telomerase should differ from other RTs in the primer grip region. In addition, the distance between motifs A and B' is longer in the TRTs than is typical for other RTs, which may represent an insertion within the "fingers" region of the structure which resembles a right hand (**Figure 3**; see Kohlstaedt et al., *supra*; Jacobo-Molina et al., *supra*; and Patel et al., *supra*).

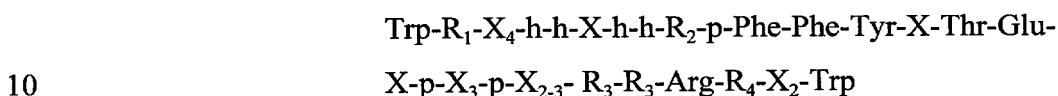
Moreover, as noted *supra*, the T motif is an additional hallmark of TRT

proteins. The T motif, shown, e.g., in **Figures 1, 4 and 11**, comprises a sequence that can be described using the formula:



- 5 where X is any amino acid and the subscript refers to the number of consecutive residues, R<sub>1</sub> is leucine or isoleucine, R<sub>2</sub> is glutamine or arginine, R<sub>3</sub> is phenylalanine or tyrosine, and R<sub>4</sub> is lysine or histidine.

The T motif can also be described using the formula:



- 10 where X is any amino acid a subscript refers to the number of consecutive residues, R<sub>1</sub> is leucine or isoleucine, R<sub>2</sub> is glutamine or arginine, R<sub>3</sub> is phenylalanine or tyrosine, R<sub>4</sub> is lysine or histidine, h is a hydrophobic amino acid selected from Ala, Leu, Ile, Val, Pro, Phe, Trp, and Met, and p is a polar amino acid selected from Gly, Ser, Thr, Tyr, Cys, Asn and Gln.

15 In one embodiment, the present invention provides isolated naturally occurring and recombinant TRT proteins comprising one or more of the motifs illustrated in **Figure 11**, e.g.,

20	Motif T	W-X <sub>12</sub> -FFY-X-TE-X <sub>10-11</sub> -R-X <sub>3</sub> -W-X <sub>7</sub> -I
	Motif T'	E-X <sub>2</sub> -V-X
	Motif 1	X <sub>3</sub> -R-X <sub>2</sub> -PK-X <sub>3</sub>
	Motif 2	X-R-X-I-X
	Motif A	X <sub>4</sub> -F-X <sub>3</sub> -D-X <sub>4</sub> -YD-X <sub>2</sub>
25	Motif B'	Y-X <sub>4</sub> -G-X <sub>2</sub> -QG-X <sub>3</sub> -S-X <sub>8</sub>
	Motif C	X <sub>6</sub> -DD-X-L-X <sub>3</sub>

When the TRT protein contains more than one TRT motif, the order (NH<sub>2</sub> → COOH) is as shown in **Figure 11**.

- 30 In one embodiment, the present invention provides isolated naturally

occurring TRT proteins comprising the following supermotif:

(NH<sub>2</sub>)- X<sub>300-600</sub>-W-X<sub>12</sub>-FFY-X-TE-X<sub>10-11</sub>-R-X<sub>3</sub>-W-X<sub>7</sub>-I-X<sub>5-20</sub>-E-X<sub>2</sub>-V-X-X<sub>5-20</sub>-X<sub>3</sub>-R-X<sub>2</sub>-  
 PK-X<sub>4-10</sub>-R-X-I-X-X<sub>60-80</sub>-X<sub>4</sub>-F-X<sub>3</sub>-D-X<sub>4</sub>-YD-X<sub>2</sub>-X<sub>80-130</sub>-Y-X<sub>4</sub>-G-X<sub>2</sub>-QG-X<sub>3</sub>-S-X<sub>8</sub>-X<sub>5-35</sub>-  
 5 X<sub>6</sub>-DD-X-L-X<sub>3</sub>-X<sub>10-20</sub>-X<sub>12</sub>-K

It will be apparent to one of skill that, provided with the reagents, including the TRT sequences disclosed herein for those reagents and the methods and guidance provided herein (including specific methodologies described *infra*) and in the  
 10 above-cited priority documents, TRT genes and proteins can be obtained, isolated and produced in recombinant form by one of ordinary skill. For example, primers (e.g., degenerate amplification primers) are provided that hybridize to gene sequences encoding RT and T motifs characteristic of TRT. For example, one or more primers or  
 15 degenerate primers that hybridize to sequences encoding the FFYXTE region of the T motif, other TRT motifs (as discussed *infra*), or combinations of motifs or consensus sequences, can be prepared based on the codon usage of the target organism, and used to amplify the TRT gene sequence from genomic DNA or cDNA prepared from the target organism. Use of degenerate primers is well known in the art and entails sets of  
 20 primers that hybridize to the set of nucleic acid sequences that can potentially encode the amino acids of the target motif, taking into account codon preferences and usage of the target organism, and by using amplification (e.g., PCR) conditions appropriate for allowing base mismatches in the annealing steps of PCR. Typically two primers are used; however, single primer (or, in this case, a single degenerate primer set) amplification systems are well known and may be used to obtain TRT genes.

25 **Table 1** provides illustrative primers of the invention that may be used to amplify novel TRT nucleic acids, particularly those from vertebrates (e.g., mammals). "N" is an equimolar mixture of all four nucleotides and sequences within parentheses are equimolar mixtures of the specified nucleotides).

**TABLE 1**  
ILLUSTRATIVE DEGENERATE PRIMERS FOR AMPLIFICATION  
OF TRT NUCLEIC ACIDS

	<u>motif</u>	<u>direction</u>	<u>5'- sequence -3'</u>
5			
10	a <u>FFYVTE</u>	Forward	TT(CT)TT(CT)TA(CT)GTNACNGA
	b <u>FFYVTE</u>	Reverse	TCNGTNAC(GA)TA(GA)AA(GA)AA
	c <u>RFIPKP</u>	Forward	(CA)GNTT(CT)AT(ACT)CCNAA(AG)CC
	d <u>RFIPKP</u>	Reverse	GG(TC)TTNGG(TGA)AT(GA)AANC
15	e <u>AYDTI</u>	Forward	GCNTA(CT)GA(CT)ACNAT
	f <u>AYDTI</u>	Reverse	TANGT(GA)TC(GA)TANGC
	g <u>GIPOG</u>	Forward	GGNAT(ACT)CCNCA(AG)GG
	h <u>GIPOGS</u>	Reverse	(GC)(AT)NCC(TC)TGNGG(TGA)ATNCC
20	i <u>LVDDFL</u>	Forward	(CT)TNGTNGA(CT)GA(CT)TT(CT)(CT)T
	j <u>DDFLLVT</u>	Reverse	GTNACNA(GA)NA(GA)(GA)AA(GA)TC(GA)TC

Allowed primer combinations (y = yes, n = no)

25		<u>Reverse</u>				
	<u>Forward</u>	<u>b</u>	<u>d</u>	<u>f</u>	<u>h</u>	<u>j</u>
	a -	n	y	y	y	y
	c -	n	n	y	y	y
	e -	n	n	n	y	y
30	g -	n	n	n	n	y
	i -	n	n	n	n	n

In one embodiment, an amplified TRT nucleic acid is used as a hybridization probe for colony hybridization to a library (e.g., cDNA library) made from the target organism, such that a nucleic acid having the entire TRT protein coding sequence, or a substantial portion thereof, is identified and isolated or cloned. Reagents and methods such as those just described were used in accordance with the methods described herein to obtain TRT gene sequences of *Oxytricha trifallax* and *Tetrahymena thermophila*, as described in detail in the priority documents. It will be recognized that following cloning of a previously uncharacterized TRT gene, the sequence can be determined by routine methods and the encoded polypeptide synthesized and assayed for a TRT activity, such as telomerase catalytic activity (as described herein and/or by telomerase assays known in the art).

It will also be apparent to those of skill that TRT genes may be cloned using any of a variety of cloning methods of the invention because the TRT motif sequences and the nucleic acids of the invention comprising such sequences can be used in a wide variety of such methods. For example, hybridization using a probe based on the sequence of a known TRT to DNA or other nucleic acid libraries from the target organism, as described in **Example 11**. It will be appreciated that degenerate PCR primers or their amplification products such as those described *supra* may themselves be labeled and used as hybridization probes. In another embodiment, expression cloning methods are used. For example, one or more antibodies that specifically bind peptides that span a TRT motif or other TRT epitope, such as the FFYXTE motif (where X is any of the twenty standard amino acids) can be employed to isolate a ribosomal complex comprising a TRT protein and the mRNA that encodes it. For generating such antibodies of the invention, the peptide immunogens are typically between 6 and 30 amino acids in length, more often about 10 to 20 amino acids in length. The antibodies may also be used to probe a cDNA expression library derived from the organism of interest to identify a clone encoding a TRT sequence. In another embodiment, computer searches of DNA databases for DNAs containing sequences conserved with known TRTs can also be used to identify a clone encoding a



TRT sequence.

In one aspect, the present invention provides compositions comprising an isolated or recombinant polypeptide having the sequence of a naturally occurring TRT protein. Usually the naturally occurring TRT has a molecular weight of between  
5 about 80,000 daltons (D) and about 150,000 D, most often between about 95,000 D and about 130,000 D. Typically, the naturally occurring TRT has a net positive charge at pH 7 (calculated pI typically greater than 9). In one embodiment, the polypeptide exhibits a telomerase activity as defined herein. In a related embodiment, the polypeptide has a TRT-specific region (T motif) sequence and exhibits a telomerase  
10 activity. The invention further provides fragments of such polypeptides. The present invention also provides isolated or recombinant polynucleotide having the sequence of a naturally occurring gene encoding a TRT protein. The invention provides isolated TRT polynucleotides having a sequence of a TRT from nonvertebrate (such as a yeast) and vertebrates, such as mammals (e.g., murine or human). The isolated polynucleotide  
15 may be associated with other naturally occurring or vector nucleic acid sequences. Typically, the isolated nucleic acid is smaller than about 300 kb, often less than about 50 kb, more often less than about 20 kb, frequently less than about 10 kb and sometimes less than about 5 kb or 2 kb in length. In some embodiments the isolated TRT polynucleotide is even smaller, such as a gene fragment, primer, or probe of less  
20 than about 1 kb or less than 0.1 kb.

### III. NUCLEIC ACIDS

#### A) GENERALLY

The present invention provides isolated and recombinant nucleic acids having a sequence of a polynucleotide encoding a telomerase catalytic subunit protein (TRT), such as a recombinant TRT gene from *Euplotes*, *Tetrahymena*, *S. pombe* or humans. Exemplary polynucleotides are provided in **Figure 13** (*Euplotes*); **Figure 15** (*S. pombe*) and **Figure 16** (human, GenBank Accession No. AF15950). The present invention provides sense and anti-sense polynucleotides having a TRT gene sequence, including probes, primers, TRT-protein-encoding polynucleotides, and the like.

#### B) HUMAN TRT

The present invention provides nucleic acids having a sequence of a telomerase catalytic subunit from humans (i.e., hTRT).

In one aspect, the invention provides a polynucleotide having a sequence or subsequence of a human TRT gene or RNA. In one embodiment, the polynucleotide of the invention has a sequence of **SEQ. ID NO: 1**, or a subsequence thereof. In another embodiment, the polynucleotide has a sequence of **SEQ. ID NO: 3** (**Figure 18**), **SEQ. ID. NO: 4** (**Figure 20\***), or subsequences thereof. The invention also provides polynucleotides with substantial sequence identity to the hTRT nucleic acid sequences disclosed herein, e.g., **SEQ. ID. NO: 1** and any others disclosed (e.g., **SEQ. ID. NOS: 4, 6** [**Figure 21**], and **7** [**Figure 22**]). Thus, the invention provides naturally occurring alleles of human TRT genes and variant polynucleotide sequences having one or more nucleotide deletions, insertions or substitutions relative to an hTRT nucleic acid sequence disclosed herein. As described *infra*, variant nucleic acids may be produced using the recombinant or synthetic methods described below or by other means.

The invention also provides isolated and recombinant polynucleotides having a sequence from a flanking region of a human TRT gene. Such polynucleotides include those derived from genomic sequences of untranslated regions of the hTRT mRNA. An exemplary genomic sequence is **SEQ. ID. NO: 6**. As described in

**Example 4, SEQ. ID. NO. 6** was obtained by sequencing a clone,  $\lambda$ G $\Phi$ 5 isolated from a human genomic library. Lambda G $\Phi$ 5 contains a 15 kilobasepair (kbp) insert including approximately 13,000 bases 5' to the hTRT coding sequences. This clone contains hTRT promoter sequences and other hTRT gene regulatory sequences (e.g., enhancers).

The invention also provides isolated and recombinant polynucleotides having a sequence from an intronic region of a human TRT gene. An exemplary intronic sequence is **SEQ. ID. NO: 7** (see **Example 3**). In some embodiments, hTRT introns are included in "minigenes" for improved expression of hTRT proteins in eukaryotic cells.

In a related aspect, the present invention provides polynucleotides that encode hTRT proteins or protein fragments, including modified, altered and variant hTRT polypeptides. In one embodiment, the encoded hTRT protein or fragment has an amino acid sequence as set forth in **SEQ ID NO: 2**, or with conservative substitutions of **SEQ ID NO: 2**. It will be appreciated that, as a result of the degeneracy of the genetic code, the nucleic acid encoding the hTRT protein need not have the sequence of a naturally occurring hTRT gene, but that a multitude of polynucleotides can encode an hTRT polypeptide having an amino acid sequence of **SEQ ID NO: 2**. The present invention provides each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices made in accordance with known triplet genetic codes, and all such variations are specifically disclosed hereby. Thus, although in some cases hTRT polypeptide-encoding nucleotide sequences that are capable of hybridizing to the nucleotide sequence of the naturally occurring sequence (under appropriately selected conditions of stringency) are preferred, it may be advantageous in other cases to produce nucleotide sequences encoding hTRT that employ a substantially different codon usage.

In particular embodiments, the invention provides hTRT oligo- and polynucleotides that comprise a subsequence of an hTRT nucleic acid disclosed herein (e.g., **SEQ ID NOS: 1, 4, 6, and 7**). The nucleic acids of the invention typically comprise at least about 10, more often at least about 12 or about 15 consecutive bases

of the exemplified hTRT polynucleotide. Often, the nucleic acid of the invention will comprise a longer sequence, such as at least about 25, about 50, about 100, about 200, or at least about 500 bases in length, for example when expression of a polypeptide is intended. In some embodiments of the present invention, the hTRT polynucleotide is  
5 other than a polynucleotide having the sequence of EST AA281296 (**SEQ. ID NO. 8**).

In still other embodiments, the present invention provides “ $\Delta$ 182 hTRT” polynucleotides having a sequence encoding naturally occurring or non-naturally occurring hTRT polynucleotides such as **SEQ ID NO: 3** or **SEQ ID NO: 4**, which do not contain the 182 basepair sequence (**SEQ ID NO: 9 [Figure 24]**) found in  
10 pGRN121 (and also absent in clone 712562). These polynucleotides are of interest, in part, because they encode polypeptides that contain different combinations of TRT motifs than found in the “full-length” hTRT polypeptide (**SEQ. ID. NO. 2**) such as is encoded by pGRN121. As discussed *infra*, it is contemplated that these polypeptides may play a biological role in nature (e.g., in regulation of telomerase expression in  
15 cells) and/or find use as therapeutics (e.g., as dominant-negative products that inhibit function of wild-type proteins), or have other roles and uses, e.g. as described herein.

For example, in contrast to the polypeptide encoded by pGRN121, clone 712562 encodes a 259 residue protein with a calculated molecular weight of approximately 30 kD (hereinafter, “712562 hTRT”). The 712562 hTRT polypeptide  
20 (**SEQ. ID NO: 10 [Figure 19]**) contains motifs T, 1, 2, and A, but not motifs B', C, D and E. Similarly, a variant hTRT polypeptide with therapeutic and other activities may be expressed from a nucleic acid similar to the pGRN121 cDNA but lacking the 182 basepairs missing in clone 712562, e.g., having the sequence **SEQ. ID. NO.: 4**. This nucleic acid (hereinafter, “pro90 hTRT”), which may be synthesized using routine  
25 synthetic or recombinant methods as described herein, encodes a protein of 807 residues (calculated molecular weight of approximately 90 kD) that shares the same amino terminal sequence as the hTRT protein encoded by **SEQ. ID. NO: 1**, but diverges at the carboxy-terminal region (the first 763 residues are common, the last 44 residues of pro90 hTRT are different than “full-length” hTRT). The pro90 hTRT  
30 polypeptide contains motifs T, 1, 2, and A, but not motifs B, C, D, E, and thus may

have some, but not all telomerase activities.

### C) PRODUCTION OF HUMAN TRT NUCLEIC ACIDS

5 The polynucleotides of the invention have numerous uses including, but not limited to, expression of polypeptides encoding hTRT or fragments thereof, use as sense or antisense probes or primers for hybridization and/or amplification of naturally occurring hTRT genes or RNAs (e.g. for diagnostic or prognostic applications), and as therapeutic agents (e.g., in antisense, triplex, or ribozyme compositions). As will be apparent upon review of the disclosure, these uses will have enormous impact on the  
10 diagnosis and treatment of human diseases relating to aging, cancer and fertility as well as the growth, reproduction and manufacture of cell-based products. As described in the following sections, the hTRT nucleic acids of the invention may be made (e.g., cloned, synthesized, or amplified) using techniques well known in the art.

#### 15 1) CLONING, AMPLIFICATION, AND RECOMBINANT PRODUCTION

In one embodiment, hTRT genes or cDNAs are cloned using a nucleic acid probe that specifically hybridizes to an hTRT mRNA, cDNA, or genomic DNA. One suitable probe for this purpose is a polynucleotide having the sequence provided in  
20 **SEQ ID NO: 1**, or a subsequence thereof. Typically, the target hTRT genomic DNA or cDNA is ligated into a vector (e.g., a plasmid, phage, virus, yeast artificial chromosome, or the like) and may be found in a genomic or cDNA library (e.g., a human placental cDNA library). Once an hTRT nucleic acid is identified, it can be isolated according to standard methods known to those of skill in the art. An illustrative  
25 example of screening a human cDNA library for the hTRT gene is provided in **Example 1**; similarly, an example of screening a human genomic library is found in **Example 4**. Cloning methods are well known and are described, for example, in Sambrook et al., *supra*; Berger and Kimmel, *supra*; Ausubel et al., *supra*; Cashion et al., U.S. Patent No. 5,017,478; and Carr, European Patent No. 0,246,864.

30 The invention also provides hTRT genomic or cDNA nucleic acids

isolated by amplification methods such as the polymerase chain reaction (PCR). In one embodiment, hTRT protein coding sequence is amplified from a RNA or cDNA sample (e.g., double stranded placental cDNA (Clontech, Palo Alto CA)) using the primers 5'-GTGAAGGCACTGTTTCAGCG-3' ("TCP1.1") and

5 5'-CGCGTGGGTGAGGTGAGGTG-3' ("TCP 1.15"). In some embodiments a third primer or second pair of primers may be used, e.g., for "nested PCR", to increase specificity. One example of a second pair of primers is 5'-CTGTGCTGGGCCTGGACGATA-3' ("billTCP6") and 5'-AGCTTGTTCTCCATGTCGCCGTAG-3' ("TCP1.14"). It will be apparent to those of

10 skill that numerous other primers and primer combinations, useful for for amplification of hTRT nucleic acids are provided by the present invention.

Moreover, the invention provides primers that amplify any specific region (e.g., coding regions, promoter regions, and/or introns) or subsequence of hTRT genomic DNA, cDNA or RNA. For example, the hTRT intron at position 274/275 of

15 **SEQ ID NO: 1** (see **Example 3**) may be amplified (e.g., for detection of genomic clones) using primers TCP1.57 and TCP1.52 (primer pair 1) or primers TCP1.49 and TCP1.50 (primer pair 2). (Primer names refer to primers listed in **Table 2**, *infra*.) The primer pairs can be used individually or in a nested PCR where primer set 1 is used first. Another illustrative example relates to primers that specifically amplify and so

20 detect the 5' end of the hTRT mRNA or the exon encoding the 5' end of hTRT gene (e.g., to assess the size or completeness of a cDNA clone). The following primer pairs are useful for amplifying the 5' end of hTRT: 1) primers K320 and K321; 2) primers K320 and TCP1.61; 3) primers K320 and K322. The primer sets can be used in a nested PCR in the order set 3, then set 2 or set 1, or set 2 then set 1. Yet another illustrative

25 example involves primers chosen to amplify or detect specifically the conserved hTRT TRT motif region comprising approximately the middle third of the mRNA (e.g., for use as a hybridization probe to identify TRT clones from nonhuman organisms). The following primer pairs are useful for amplifying the TRT motif region of hTRT nucleic acids: primers K304 and TCP1.8 (primer pair 6), or primers LT1 and TCP1.15 (primer

30 pair 7). The primer sets can be used in a nested PCR experiment in the order set 6 then

set 7.

Suitable PCR amplification conditions are known to those of skill and include (but are not limited to) 1 unit Taq polymerase (Perkin Elmer, Norwalk CT), 100  $\mu$ M each dNTP (dATP, dCTP, dGTP, dTTP), 1x PCR buffer (50 mM KCl, 10 mM Tris, pH 8.3 at room temperature, 1.5 mM  $MgCl_2$ , 0.01% gelatin) and 0.5  $\mu$ M primers, with the amplification run for about 30 cycles at 94° for 45 sec, 55° for 45 sec and 72° for 90 sec. It will be recognized by those of skill in the art that other thermostable DNA polymerases, reaction conditions, and cycling parameters will also provide suitable amplification. Other suitable *in vitro* amplification methods that can be used to obtain hTRT nucleic acids include, but are not limited to, those herein, *infra*. Once amplified, the hTRT nucleic acids can be cloned, if desired, into any of a variety of vectors using routine molecular biological methods or detected or otherwise utilized in accordance with the methods of the invention.

One of skill will appreciate that the cloned or amplified hTRT nucleic acids obtained as described above can be prepared or propagated using other methods, such as chemical synthesis or replication by transformation into bacterial systems such as *E. coli* (see, e.g., Ausubel et al., *supra*) or eukaryotic, such as mammalian, expression system. Similarly, hTRT RNA can be expressed in accordance with the present *in vitro* methods, or in bacterial systems such as *E. coli* using, for example, commercially available vectors containing promoters recognized by an RNA polymerase such as T7, T3 or SP6, or transcription of DNA generated by PCR amplification using primers containing an RNA polymerase promoter.

The present invention further provides altered or modified hTRT nucleic acids. It will be recognized by one of skill that the cloned or amplified hTRT nucleic acids obtained can be modified (e.g., truncated, derivatized, altered) by methods well known in the art (e.g., site-directed mutagenesis, linker scanning mutagenesis) or simply synthesized de novo as described below. The altered or modified hTRT nucleic acids are useful for a variety of applications, including, but not limited to, facilitating cloning or manipulation of an hTRT gene or gene product, or expressing a variant hTRT gene product. For example, in one embodiment, the hTRT gene sequence is

altered such that it encodes an hTRT polypeptide with altered properties or activities, as discussed in detail in *infra*, for example, by mutation in a conserved motif of hTRT. In an other illustrative example, the mutations in the protein coding region of an hTRT nucleic acid may be introduced to alter glycosylation patterns, to change codon  
5 preference, to produce splice variants, remove protease-sensitive sites, create antigenic domains, modify specific activity, and the like). In other embodiments, the nucleotide sequence encoding hTRT and its derivatives is changed without altering the encoded amino acid sequences, for example, the production of RNA transcripts having more desirable properties, such as increased translation efficiency or a greater or a shorter  
10 half-life, compared to transcripts produced from the naturally occurring sequence. In yet another embodiment, altered codons are selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency with which particular codons are utilized by the host. Useful *in vitro* and *in vivo* recombinant techniques that can be used to prepare  
15 variant hTRT polynucleotides of the invention are found in Sambrook et al. and Ausubel et al., both *supra*.

As noted *supra*, the present invention provides nucleic acids having flanking (5' or 3' ) and intronic sequences of the hTRT gene. The nucleic acids are of interest, *inter alia*, because they contain promoter and other regulatory elements  
20 involved in hTRT regulation and useful for expression of hTRT and other recombinant proteins or RNA gene products. It will be apparent that, in addition to the nucleic acid sequences provided in **SEQ. ID NOS: 6 and 7**, additional hTRT intron and flanking sequences may be readily obtained using routine molecular biological techniques. For example, additional hTRT genomic sequence may be obtained by further sequencing of  
25 Lambda clone GΦ5, described *supra* and in **Example 4**. Still other hTRT genomic clones and sequences may be obtained by screening a human genomic library using an hTRT nucleic acid probe having a sequence or subsequence from **SEQ. ID. NO. 1**. Additional clones and sequences (e.g., still further upstream) may be obtained by using labeled sequences or subclones derived from λGΦ5 to probe appropriate libraries.  
30 Other useful methods for further characterization of hTRT flanking sequences include



those general methods described by Gobinda et al., 1993, *PCR Meth. Applic.* 2:318; Triglia et al., 1988, *Nucleic Acids Res.* 16:8186; Lagerstrom et al., 1991, *PCR Methods Applic.* 1:111; and Parker et al., 1991, *Nucleic Acids Res.* 19:3055.

Intronic sequences can identified by routine means such as by  
5 comparing the hTRT genomic sequence with hTRT cDNA sequences (see, e.g.,  
**Example 3**), by S1 analysis (see Ausubel et al., *supra*, at Chapter 4), or various other  
means known in the art. Intronic sequences can also be found in pre-mRNA (i.e.,  
unspliced or incompletely spliced mRNA precursors), which may be amplified or  
cloned following reverse transcription of cellular RNA.

10 When desired, the sequence of the cloned, amplified, or otherwise  
synthesized hTRT or other TRT nucleic acid can be determined or verified using DNA  
sequencing methods well known in the art (see, e.g., Ausubel et al., *supra*). Useful  
methods of sequencing employ such enzymes as the Klenow fragment of DNA  
polymerase I, Sequenase (US Biochemical Corp, Cleveland OH), *Taq* DNA polymerase  
15 (Perkin Elmer, Norwalk CT), thermostable T7 polymerase (Amersham, Chicago IL), or  
combinations of recombinant polymerases and proofreading exonucleases such as the  
ELONGASE Amplification System marketed by Gibco BRL (Gaithersburg MD).  
When sequencing or verifying the sequence of oligonucleotides (such as  
oligonucleotide made *de novo* by chemical synthesis), the method of Maxam and  
20 Gilbert may be preferred (Maxam and Gilbert, 1980, *Meth. Enz.* 65:499; Ausubel et al.,  
*supra*, Ch. 7).

The 5' untranslated sequences of hTRT or other TRT mRNAs can be  
determined directly by cloning a "full-length" hTRT or other cDNA using standard  
methods such as reverse transcription of mRNA, followed by cloning and sequencing  
25 the resulting cDNA. Preferred oligo(dT)-primed libraries for screening or amplifying  
full length cDNAs that have been size-selected to include larger cDNAs may be  
preferred. Random primed libraries are also suitable and often include a larger  
proportion of clones that contain the 5' regions of genes. Other well known methods  
for obtaining 5' RNA sequences, such as the RACE protocol described by Frohman et  
30 al., 1988, *Proc. Nat. Acad. Sci USA* 85:8998, may also be used. If desired, the

transcription start site of an hTRT or other TRT mRNA can be determined by routine methods using the nucleic acids provided herein (e.g., having a sequence of **SEQ. ID. NO: 1**). One method is S1 nuclease analysis (Ausubel et al., *supra*) using a labeled DNA having a sequence from the 5' region of **SEQ ID NO: 1**.

5

## 2) CHEMICAL SYNTHESIS OF NUCLEIC ACIDS

The present invention also provides hTRT polynucleotides (RNA, DNA or modified) that are produced by direct chemical synthesis. Chemical synthesis is generally preferred for the production of oligonucleotides or for oligonucleotides and polynucleotides containing nonstandard nucleotides (e.g., probes, primers and antisense oligonucleotides). Direct chemical synthesis of nucleic acids can be accomplished by methods known in the art, such as the phosphotriester method of Narang et al., 1979, *Meth. Enzymol.* 68:90; the phosphodiester method of Brown et al., *Meth. Enzymol.* 68:109 (1979); the diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.*, 15 22:1859 (1981); and the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis typically produces a single stranded oligonucleotide, which may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase and an oligonucleotide primer using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is often limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences or by more elaborate synthetic methods.

It will be appreciated that the hTRT (or hTR or other) polynucleotides and oligonucleotides of the invention can be made using nonstandard bases (e.g., other than adenine, cytidine, guanine, thymine, and uridine) or nonstandard backbone structures to provides desirable properties (e.g., increased nuclease-resistance, 25 tighter-binding, stability or a desired  $T_M$ ). Techniques for rendering oligonucleotides nuclease-resistant include those described in PCT publication WO 94/12633. A wide variety of useful modified oligonucleotides may be produced, including oligonucleotides having a peptide-nucleic acid (PNA) backbone (Nielsen et al., 1991, 30 *Science* 254:1497) or incorporating 2'-O-methyl ribonucleotides, phosphorothioate

nucleotides, methyl phosphonate nucleotides, phosphotriester nucleotides, phosphorothioate nucleotides, phosphoramidates. Still other useful oligonucleotides may contain alkyl and halogen-substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH<sub>3</sub>, F, OCN, OCH<sub>3</sub>OCH<sub>3</sub>, OCH<sub>3</sub>O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub> or O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub> where n is from 1 to about 10; C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF<sub>3</sub>; OCF<sub>3</sub>; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH<sub>3</sub>; SO<sub>2</sub>CH<sub>3</sub>; ONO<sub>2</sub>; NO<sub>2</sub>; N<sub>3</sub>; NH<sub>2</sub>; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a cholesteryl group; a folate group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Folate, cholesterol or other groups which facilitate oligonucleotide uptake, such as lipid analogs, may be conjugated directly or via a linker at the 2' position of any nucleoside or at the 3' or 5' position of the 3'-terminal or 5'-terminal nucleoside, respectively. One or more such conjugates may be used. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other embodiments may include at least one modified base form or "universal base" such as inosine, or inclusion of other nonstandard bases such as queosine and wybutosine as well as acetyl-, methyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases. The invention further provides oligonucleotides having backbone analogues such as phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, chiral-methyl phosphonates, nucleotides with short chain alkyl or cycloalkyl intersugar linkages, short chain heteroatomic or heterocyclic intersugar ("backbone") linkages, or CH<sub>2</sub>-NH-O-CH<sub>2</sub>, CH<sub>2</sub>-N(CH<sub>3</sub>)-OCH<sub>2</sub>, CH<sub>2</sub>-O-N(CH<sub>3</sub>)-CH<sub>2</sub>, CH<sub>2</sub>-N(CH<sub>3</sub>)-N(CH<sub>3</sub>)-CH<sub>2</sub> and O-N(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub> backbones (where phosphodiester is O-P-O-CH<sub>2</sub>), or mixtures of the same. Also useful are oligonucleotides having morpholino backbone structures (U.S. Patent No. 5,034,506).

Useful references include Oligonucleotides and Analogues, A Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan et al., 9 July 1993, J. Med. Chem. 36(14):1923-1937; Antisense Research and Applications (1993, CRC Press), in its entirety and specifically Chapter 15, by Sanghvi, entitled "Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides." Antisense Therapeutics, ed. Sudhir Agrawal (Humana Press, Totowa, New Jersey, 1996)

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#### D) LABELING NUCLEIC ACIDS

It is often useful to label the nucleic acids of the invention, for example, when the hTRT or other oligonucleotides or polynucleotides are to be used as nucleic acid probes. The labels (see *infra*) may be incorporated by any of a number of means well known to those of skill in the art. In one embodiment, an unamplified nucleic acid (e.g., mRNA, polyA mRNA, cDNA) is labeled. Means of producing labeled nucleic acids are well known to those of skill in the art and include, for example, nick-translation, random primer labeling, end-labeling (e.g. using a kinase), and chemical conjugation (e.g., photobiotinylation). In another embodiment, the label is simultaneously incorporated during an amplification step in the preparation of the sample nucleic acids. Thus, for example, polymerase chain reaction (PCR) or other nucleic acid amplification method with labeled primers or labeled nucleotides will provide a labeled amplification product. In another embodiment, transcription amplification using a labeled nucleotide (e.g. fluorescein-labeled UTP and/or CTP) incorporates a label into the transcribed nucleic acids. An amplification product may also, or alternatively, be labeled after the amplification is completed.

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#### E) ILLUSTRATIVE OLIGONUCLEOTIDES

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As noted *supra* and discussed in detail *infra*, oligonucleotides are used

for a variety of uses including as primers, probes, therapeutic or other antisense oligonucleotides, triplex oligonucleotides, and numerous other uses as apparent from this disclosure. Table 1 provides certain illustrative specific oligonucleotides that may be used in the practice of the invention. It will be appreciated that numerous other  
5 useful oligonucleotides of the invention may be synthesized by one of skill, following the guidance provided herein.

In **Table 2**, “seq” means that the primer has been used, or is useful, for sequencing; “PCR” means that the primer has been used, or is useful, for PCR; “AS” means that means that the primer has been used, or is useful for antisense inhibition of  
10 telomerase activity; “CL” means that the primer has been used, or is useful in cloning regions of hTERT genes or RNA, “mut” means that the primer has been used, or is useful for constructing mutants of hTERT genes or gene products. “UC” means “upper case,” and “lc” means “lower case.” Mismatches and insertions (relative to SEQ ID NO: 1) are indicated by underlining; deletions are indicated by a “-”. It will be  
15 appreciated that nothing in **Table 2** is intended to limit the use of any particular oligonucleotide to any single use or set of uses.

TABLE 2  
USEFUL OLIGONUCLEOTIDES

primer	5'-sequence-3' *	Notes	mismatch? *	USE			
				seq	PCR	AS	MUT
TCP1.1	GTGAAGGCACTGTTACGCG			X	X		
TCP1.2	GTGGATGATTCTTGTGG			X	X		
TCP1.4	CTGGACACTCAGCCCTGG			X	X		
TCP1.5	GGCAGGTGTCTGGACACT			X	X		
TCP1.6	TTTGATGATGCTGGCGATG			X	X		
TCP1.7	GGGGCTCGTCTTCTACAGG		Y	X	X		
TCP1.8	CAGCAGGAGGATCTTGTAG			X	X		
TCP1.9	TGACCCAGGAGTGGCAGG			X	X		
TCP1.10	TCAAGCTGACTCGACACCG			X	X		
TCP1.11	CGGCGTGACAGGGCTGC			X	X		
TCP1.12	GCTGAAGGCTGAGTGCC			X	X		
TCP1.13	TAGTCCATGTTCACAATCG			X	X		
TCP1.14	CTGTGCTGGGCTGGACGATA			X	X		
TCP1.15	CGCGTGGTGAGGTGAGGTG			X	X		
TCP1.16	TTTCCGTGTGAGTGTTTC			X	X		
TCP1.17	GTCACCGTGTGGGCAGG			X	X		
TCP1.19	GCTACCTGCCCAACACGG			X	X		
TCP1.20	GCGCGAAGAACGTGCTGG			X	X		
TCP1.21	CA-CTGCTCCTTGTGCGCTG		Y	X	X		
TCP1.22	TTCCCAAGGACTTTGTTGC			X	X		
TCP1.24	TGTTCTCAAGACGCACTG		Y	X	X		
TCP1.25	TACTGCGTGCCTCGGTATG			X	X		
TCP1.26	GGTCTTGGCGCTGAAGTGT			X	X		
TCP1.27	TGGTTCACCTGCTGGCACG			X	X		
TCP1.28	GTGGTTTCTGTGTGGTGTC			X	X		
TCP1.29	GACACCACACAGAAACCAC			X	X		
TCP1.30	GTGCCAGCAGGTGAACCAG			X	X		
TCP1.32B	GCAGTGGCTCTTGAGGAGC			X	X		
TCP1.33	TGGAACCATAGCGTCAGGGAG			X	X		
TCP1.34	GGCTCCCTGACGCTATGGTT			X	X		
TCP1.35	GC(GT)CGGCGCTGCCACTCAGG			X	X		
TCP1.35t	GCTCGGCGCTGCCACTCAGG						
TCP1.36	ACGCCGAGACCAAGCACCTTC			X			X

TABLE 2

(cont.)

TCP1.38	CCAAAGAGGTGGCTTCTCG	X	X
TCP1.39	AAGCCAGCACGTTCTTCG	X	X
TCP1.40	CACGTTCTGGCGGCGCTG	X	X
TCP1.41	CCTTACCAACGAGCGTGG	X	X
TCP1.42	GGCGACGACGTGCTGGTTC	X	X
TCP1.43	GGCTAGGGGACAGGCCAC	X	X
TCP1.44	CTGGCAGGTGTACGGCTTC	X	X
TCP1.45	CGGTGGACCGAGTGACCGTGGTTC	X	X
TCP1.46	GACGTGGTGGCGGATGTGG	X	X
TCP1.47	GAAGTCTGCCGTGCCCCAAGAG	X	X
TCP1.48	GACACCACACAGAAACCACGGTCAC	X	X
TCP1.49	CGCCCCCTCTTCCGCCAGGT	X	X
TCP1.50	CGAAGCCGAAGGCCAGCACGTTCTT	X	X
TCP1.51	GGTGGCCGAGTGTGCAGAGG	X	X
TCP1.52	GTAGTGGCACGCTGGTGTGAAG	X	X
TCP1.53	TGGGCGACGACGTGCTGGTICA	X	X
TCP1.54	TATGTTCCAGGCCGTTCCGATCC	X	X
TCP1.55	CCAGCTGGCCTACAGGTGTGC	X	X
TCP1.56	GGCCTCCCTGACGCTATGTTCCAG	X	X
TCP1.57	GGTGTGCCGCTGGCCACGTTGG	X	X
TCP1.58	TCCCAGGGCACGCACACAGGCACT	X	X
TCP1.59	GTACAGGGCACACCTTTGGTCACTC	X	X
TCP1.60	TCGACGACGTACACACTCATCAGCC	X	X
TCP1.61	AGGGCAGCACCTCGCGGTAGTGGC	X	X
TCP1.62	CCACCAGTCTCTCAGGCAGGACAC	X	X
TCP1.63	CCAGGGCTCCCACTGCGCAGCAG	X	X
TCP1.64	CGCACGAACGTGGCCAGCGGCAGCA	X	X
TCP1.65	TGACCGTGGTTTCTGTGTGTGT	X	X
TCP1.66	CCCTCTTCAAGTGTGTCTGATTCC	X	X
TCP1.67	ATCGGGCCACCACGTCCT	X	X
TCP1.68	TGCTCCAGACACTCGCGCGTAGAA	X	X
TCP1.69	ACGAAGCCGTACACCTGCC	X	X
TCP1.72	CGACATCCCTGCGTCTTGGCTTTC	X	X
TCP1.73	CACTGTGGCTCATTCAGGG	X	X
TCP1.74	GCGACATGGAGAACAAAGC	X	X
TCP1.75	GCAGCCATACTCAGGGACAC	X	X

TABLE 2

(cont.)

TCP1.76	CCATCCTCTCCACGCTGCTC	X	X	X	
TCP1.77	GCGATGACCTCCGTGAGCTG	X	X	X	
TCP1.78	CCCAGGACAGGCTCACGGA	X	X	X	
biITCP1	CCTCTCAAGTGCTGTCTGATTCC	X	X	X	
biITCP2	CAGCTCGACGACGTACACACTCATC	X	X	X	
biITCP4	CTGACGTCCAGACTCCGCTTCAT	X	X	X	
biITCP6	AGCTTGTTCTCCATGTGCGCGTAG	X	X	X	
rprim01	GACCTGAGCAGCTCGACGACGTACACACTCATC	X	X	X	
L11	GTCGTGAGCTGCTCAGGTC	X	X	X	
L12	AGCACGCTGAACAGTGCCCT	X	X	X	
L13	GACCTGAGCAGCTCGACGAC	X	X	X	
L14	AAGGCACCTGTTACGGTGCT	X	X	X	
L15	CGGCCGAGTGCTGGAGCAA	X	X	X	
L16	GGATGAAGCGGAGTCTGGA	X	X	X	
BamHI1L17	ATGGAICCGTCTCGAGCTGCTCAGGTCT				X
Sal11L18	ATCAGC TGAGCACGCTGAACAGTGCCCTC				X
K303	GTCTCCGTGACATAAAAGAAAGAC	X	X	X	
K304	GCCAAAGTTCCTGCACTGGCT	X	X	X	
K305	GCCTGTTCTTTTGAAACGTGGTCT	X	X	X	
K306	XXGCCTGTTCTTTTGAAACGTGGTCT	X	X	X	
K311	GTCAAAGATGCCTGAGATAGAAC	X	X	X	
K312	TGCTTAGCTTGTGGGGGTGTCA	X	X	X	
K313	TGCTTAGCTTGTGGGGGTGTCA	X	X	X	
K320	GCTGCTCCTGTGCGCACGT	X	X	X	
K321	CAGCGGGAGCGCGCGGCATC	X	X	X	
K322	TGGGCCACCAAGCGCGCGGAAA	X	X	X	
slanti.1	CGGCGCAGCCCCGTCAAGCTTGGGG		Y		
slanti.2	CCGACAGCTCCCGCAGCTGCACCC		Y		
slanti.3	CGTACACATCATCAGCCAGTGCAGGAACCTTGGC	X	X	X	
slanti.4	CGGCGCGCTCTAGTTAGCACGCTGAACAGTGCCTTC	X	X	X	
slanti.5	GCGGAGTCTGGACGTACAGGGCGGCTGGCTTCCCG	X	X	X	
UTR2	ATTTGACCCACAGGACCCCATCCAG	X	X	X	
FW5	ATGACCGCCCTCCTCGTGAG	X	X	X	
Nam1	GCCACCCCGCGATGCC	X	X	X	
Nam2	AGCCTTGGCCCCGGCCA	X	X	X	

BamHI site  
Pvu II site (not Sal I)

X = biotin, = K305



## TABLE 2

**(cont.)**

	TCCCACGTGCGCAGCAG	Xba I site / HA tag / hTRT into pGRN121 inserts HA tag into a Nde I site at 5' end of hTRT		
Nam3	AGCAGGACGCAGCGCTG		X	X
Nam4	CGCGGTAGTGCTGCGCAGCAGGGAGCGCACGGC		X	X
PE01	CCAGGGCTTCCACGTGCGCAGCAGGACGCAGCGC		X	X
PE02	CTAGTCTAGATCA / GCTAGCGTAATCTGGAACATCGTA		X	X
LM101	TGGGTA / GTCCAGGATGGTCTTTGAAGTC			
LM103	TACCATGGGCTACCCATACGACGTTCCAGATTACGCTCA			
LM104	TATGAGCGTAATCTGGAACGTCGTATGGGTAGCCCCATGG			
LM105	GTGTACGTGTCGAGCTCCTCAGGCTCGCCTTTT			
LM106	ATGTCACGGAG			
LM107	GTGTACGTGTCGAGCTCCTCAGGCTTTTCGCTTATGTC			
LM108	ACGGAGACC			
LM109	CCTCAGGCTTTTCTTTGCTGTCAACGAGACAACGTTTT			
LM_FFYT	CAAAAGAACAG			
TCP061:	GGTCTTTCTTTTATGTCGGGAGACAACGTTT			
HUM01:	CAAAAGAACAG			
HUM02:	CTTTCTTTATGTCACGGCGACAACGTTTCAAAGAACA			
HUM03:	ATGAGTGTGACGTCGTCGAGCTCCTCAGGCTTACCACG			
HUM04:	CAAAAGAACAGGCTCTTTTC			
SLW FIN	GGCTGATGAGTGTGACGTCGTCGA			
SLW FIC	ACGTGGTCTCCGTGACATAAAAAGA			
SLW F2N	AGGTCTTTCTTTTATGTCACGGA			
SLW F1N	CACAGACCCCGTCGCCCTGGTC			
SLW F1C	CGGAGTCTGGACGTCAGCAGGGC			
SLW F2M	cgcggaaccctactaaAATGCGCGCGCTCCCCCGCTGC			
SLW F2L	cgcggaattcgtagtactaaCAAAAGAGAGGTGGCTTCTTCGGC			
SLW F2O	SLW FIN / SLW F1C amplify a 893 nt piece of pGRN121 (782 to 1636)			
SLW F2P	cgcggaaccctactaaAGCCACCTCTTTGGAGGGTGCG			
SLW F2Q	for GST fusion construct (1625 to 2458)			

TABLE 2

(cont.)

SLW F2C	<u>ccggaattcgtaactta</u> AGACCTGAGCAGCTCGACGAC	UC = hTRT seq, lc = BamHI site + 2 stop codons for GST fusion construct (1625 to 2458)	X	X
SLW F3N	SLW F2N / SLW F2C amplify a 872 nt piece of pGRN121 (1625 to 2458) <u>cgcggaatccgtaactaaa</u> ATGAGTGTGTACGTCGTCGAG	UC = hTRT seq, lc = EcoRI site + 3 stop codons		
SLW F3C	<u>ccggaattcgtaactta</u> GATCCCTGGCACTGGACG	for GST fusion construct (2426 to 3274) UC = hTRT seq, lc = BamHI site + 2 stop codons for GST fusion construct (2426 to 3274) UC = hTRT seq, lc = EcoRI site + 3 stop codons	X	X
SLW F4N	SLW F3N / SLW F3C amplify a 887 nt piece of pGRN121 (2426 to 3274) <u>cgcggaatccgtaactaaa</u> ATCCCGCAGGGCTCCATCCTC	for GST fusion construct (3272 to 4177) UC = hTRT seq, lc = BamHI site + 2 stop codons for GST fusion construct (3272 to 4177) UC = hTRT seq, lc = EcoRI site + 3 stop codons	X	X
SLW F4C	<u>ccggaattcgtaactta</u> GTCACGAGATGGTCTTGAAGTC		X	X
40-60	SLW F4N / SLW F4C amplify a 944 nt piece of pGRN121 (3272 to 4177) GGCATCGCGGGGGTGGCCGGG			X
260-280	GGACACCTGGCGGAAGGAGGG	phosphorothioate		X
500-520	GCGTGCCAGCAGGTGAACCCAG	phosphorothioate		X
770-790	CTCAGGGGCAGCGCCACGCCT	phosphorothioate		X
885-905	AGGTGGCTTCTCGCGGGTC	phosphorothioate		X
1000-1020	GGACAAAGCGTGTCCAGGGA	phosphorothioate		X
1300-1320	GCTGGGTGACCGCAGCTCGC	phosphorothioate		X
1520-1540	GATGAACCTTCTTGGTTCCT	phosphorothioate		X
2110-2130	GTGCGCCAGCCCTGTGGATA	phosphorothioate		X
2295-2315	GCCCATGGCGGCCCTTCTGGA	phosphorothioate		X
2450-2470	GAGGCCACTGCTGGCCTCAT	phosphorothioate		X
2670-2690	GGGTGAGGTGAGGTGCACCA	phosphorothioate		X
3080-3110	GCTGCAGCACACATCGGTGAACCTGTACGC	phosphorothioate		X
3140-3160	GACGCGCAGGAAAATGTGGG	phosphorothioate		X
3690-3710	CCGAGGCCAGCCTGTGGGA	phosphorothioate		X
s1	GCGACGACTGACATTGGCCGG	phosphorothioate		X
s2	GGCTCGAAGTAGCACCGGTGC	phosphorothioate, control oligo		X
s3	GTGGGAACAGCCGATGTCCC	phosphorothioate, control oligo		X
55-75	CAGCGGGGAGCGCGGGCATC	phosphorothioate, control oligo		X
151-171	CAGCACCTCGCGGTAGTGGCT	phosphorothioate		X
TP1.1	TCAAGCCAAACCTGAATCTGAG	phosphorothioate		X

TABLE 2

(cont.)

TP1.2	CCCGAGTGAATCTTTCTACGC	X
TP1.3	GTCTCTGGCAGTTCTCATCCC	X
TP1.4	TTTAGGCATCCTCCCAAGCACA	X

#### IV. TRT PROTEINS AND PEPTIDES

##### A) GENERALLY

The invention provides a wide variety of hTRT proteins useful for, *inter alia*, inhibition of telomerase activity in a cell, induction of an anti-hTRT immune reponse, as a therapeutic reagent, as a standard or control in a diagnostic assay, as a target in a screen for activation or inhibition of an activity of hTRT or telomerase, and numerous other uses that will be apparent to one of skill or which are described herein. The hTRT proteins of the invention include functionally active proteins (useful for e.g., conferring telomerase activity in a telomerase-negative cell) and variants, inactive variants (useful for e.g., inhibiting telomerase activity in a cell), hTRT polypeptides, proteins, and telomerase RNPs (e.g., ribonucleoprotein complexes comprising the proteins) that exhibit one, several, or all of the functional activities of naturally occurring hTRT and telomerase, as discussed in greater detail for illustrative purposes, below.

In one embodiment, the hTRT protein of the invention is a polypeptide having a sequence of **SEQ. ID. NO: 2** [Figure 17], or a fragment thereof. In another embodiment, the hTRT polypeptide differs from **SEQ. ID. NO: 2** by internal deletions, insertions, or conservative substitutions of amino acid residues. In a related embodiment, the invention provides hTRT polypeptides with substantial similarity to **SEQ. ID. NO: 2**. The invention further provides hTRT polypeptides that are modified, relative to the amino acid sequence of **SEQ. ID. NO: 2**, in some manner, e.g., truncated, mutated, derivatized, or fused to other sequences (e.g., to form a fusion protein). Moreover, the present invention provides telomerase RNPs comprising an hTRT protein of the invention complexed with a template RNA (e.g., hTR). In other embodiments, one or more telomerase-associated proteins is associated with hTRT protein and/or hTR.

The invention also provides other naturally occurring hTRT species or nonnaturally occurring variants, such as proteins having the sequence of, or substantial similarity to **SEQ. ID NO: 5** [Figure 19], **SEQ. ID. NO. 10** [Figure 19], and

fragments, variants, or derivatives thereof.

The invention provides still other hTRT species and variants. One example of a hTRT variant may result from ribosome frameshifting of mRNA encoded by the clone 712562 (SEQ. ID. NO: 3 [Figure 18]) or the pro90 variant hTRT shown in SEQ. ID. NO: 4 [Figure 20] and so result in the synthesis of hTRT polypeptides containing all the TRT motifs (for a general example, see, e.g., Tsuchihashi et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:2516; Craigengen et al., 1987, *Cell* 50:1; Weiss, 1990, *Cell* 62:117). Ribosome frameshifting can occur when specific mRNA sequences or secondary structures cause the ribosome to "stall" and jump one nucleotide forwards or back in the sequence. Thus, a ribosome frameshift event on the 712562 mRNA could cause the synthesis of an approximately 523 amino acid residue polypeptide. A ribosome frameshift event on the pro90 sequence could result in a protein with approximately 1071 residues. It will be appreciated that proteins resulting from ribosome frameshifting can also be expressed by synthetic or recombinant techniques provided by the invention.

Human TRT proteins, peptides, and functionally equivalent proteins may be obtained by purification, chemical synthesis, or recombinant production, as discussed in greater detail below.

## B) TRT PROTEIN ACTIVITIES

The TRT polypeptides of the invention (including fragments, variants, products of alternative alleles, and fusion proteins) can have one or more or all of the functional activities associated with native hTRT. Except as noted, as used herein, an hTRT or other TRT polypeptide is considered to have a specified activity if the activity is exhibited by either the hTRT protein without an associated RNA (e.g., hTR) or in an hTRT-hTR complex. The hTR-binding activity of hTRT is one example of an activity associated with the hTRT protein. Methods for producing complexes of nucleic acids (e.g., hTR) and the hTRT polypeptides of the invention are described *infra*.

Modification of the hTRT protein (e.g., by chemical or recombinant means, including mutation or modification of a polynucleotide encoding the hTRT

polypeptide or chemical synthesis of a polynucleotide that has a sequence different than a native polynucleotide sequence) to have a different complement of activities than native hTERT may be useful in therapeutic applications or in screening for specific modulators of hTERT or telomerase activity. In addition, assays for various hTERT activities may be particularly useful for identification of agents (e.g., activity modulating agents) that interact with hTERT or telomerase to change telomerase activity.

The activities of native hTERT, as discussed *infra*, include telomerase catalytic activity (which may be either processive or non-processive activity); telomerase processivity; conventional reverse transcriptase activity; nucleolytic activity; primer or substrate (telomere or synthetic telomerase substrate or primer) binding activity; dNTP binding activity; RNA (i.e., hTR) binding activity; and protein binding activity (e.g., binding to telomerase-associated proteins, telomere-binding proteins, or to a protein-telomeric DNA complex). It will be understood, however, that present invention also provides hTERT compositions without any particular hTERT activity but with some useful activity related to the hTERT or other TRT proteins (e.g., certain short immunogenic peptides, inhibitory peptides).

### 1) TELOMERASE CATALYTIC ACTIVITY

As used herein, a polypeptide of the invention has "telomerase catalytic activity," when the polypeptide is capable of extending a DNA primer or substrate by adding a partial, one, or more than one repeat of a sequence (e.g., TTAGGG) encoded by a template nucleic acid (e.g., hTR). This activity may be processive or nonprocessive. Processive activity occurs when a telomerase RNP adds multiple repeats to a primer or telomerase before the DNA is released by the enzyme complex. Non-processive activity occurs when telomerase adds a partial, or one, repeat to a primer and is then released. *In vivo*, however, a non-processive reaction acid multiple repeats by successive rounds of association, extension, and dissociation. This can occur *in vitro* as well, but it is not observed in standard assays due to the vastly large molar excess of primer over telomerase in standard assay conditions.

To characterize an hTERT polypeptide as having non-processive activity, a

conventional telomerase reaction is performed using conditions that favor a non-processive reaction, for example high temperatures (35-40°C), low dGTP concentrations (1  $\mu$ M or less), high primer concentrations (5  $\mu$ M or higher), and high dATP/TTP concentrations (2 mM or higher), with the temperature and dGTP typically having the greatest effect. To characterize a hTERT polypeptide as having processive activity, a conventional telomerase reaction is performed using conditions that favor a processive reaction (for example, 27-30°C), high dGTP concentration (10  $\mu$ M or higher), low primer concentration (1  $\mu$ M or lower), and low dATP,TTP concentration (0.3-1 mM) with the temperature and dGTP typically concentration being the most critical. Alternatively, a TRAP assay (for processive or moderately processive activity) or the dot-blot and gel blot assays (for processive activity) may be used. The hTERT polypeptide of the invention can possess a non-processive activity, but not a processive activity (e.g., if an alteration of the hTERT polypeptide reduces or eliminates the ability to translocate), may be solely processive, or may possess both activities.

a) Non-processive Activity

A non-processive telomerase catalytic activity can extend the DNA primer from the position where the 3' end anneals to the RNA template to the 5' end of the template, typically terminating with the addition of the first G residue (as, for example, when the template is hTR). As shown in **Table 3**, the exact number of nucleotides added is dependent on the position of the 3' terminal nucleotide of the primer in the TTAGGG repeat sequence.

**TABLE 3**  
NONPROCESSIVE ACTIVITY

- i)       -----TTAGGGttag (DNA)  
          3'-----AUCCCAAUC-----5' (RNA)
- ii)       -----TTAGggttag (DNA)  
          3'-----AUCCCAAUC-----5' (RNA)

In DNA, UC = primer, lc = added nucleotides

Thus, 4 nucleotides are added to the --TTAGGG primer (i) while 6 nucleotides are added to the --TTAG primer (ii). The first repeat added by telomerase in a processive reaction is equivalent to this step; however, in a processive reaction telomerase performs a translocation step where just the 3' end is released and rebound at the 3' region of the template in a position sufficient to prime addition of another repeat (see Morin, 1997, *Eur. J. Cancer* 33:750).

A fully non-processive reaction produces only one band in a conventional assay using a single synthetic primer. Because this result could also be produced by other enzymes, such as a terminal transferase activity, it may be desirable in some applications to verify that the product is a result of a telomerase catalytic activity. A telomerase (hTRT) generated band can be distinguished by several additional characteristics. The number of nucleotides added to the end of the primer should be consistent with the position of the primer terminus. Thus, a --TTAGGG primer should have 4 nucleotides added and a --TTAG primer should have 6 nucleotides added (see above). In practice, two or more sequence permuted primers can be used which have the same overall length but different 5' and 3' endpoints. As an illustrative example, the non-processive extension of primers TTAGGGTTAGGGTTAGGG and GTTAGGGTTAGGGTTAGG will generate products whose absolute length will be one nucleotide different (4 added to TTAGGGTTAGGGTTAGGG for a 22 nt total length, and 5 added to GTTAGGGTTAGGGTTAGG for a 23 nt total length). The nucleotide dependence of the reaction should be consistent with the position of the primer terminus. Thus, a --TTAGGG primer product should require dGTP, TTP, and dATP, but not dCTP, and a ---AGGGTT primer product should require dGTP and dATP, but not TTP or dCTP. The activity should be sensitive to RNAase or micrococcal nuclease pre-treatment (see Morin, 1989, *Cell* 59: 521) under conditions that will degrade hTR and so eliminate the template.



## b) Processive Activity

In practice, a processive activity is easily observed by the appearance of a six nucleotide ladder in a conventional assay, TRAP assay, gel-blot assay or the dot-blot assay. The conventional assay is described in Morin, 1989, *Cell* 59:521, which is incorporated herein in its entirety and for all purposes. The TRAP assay is described in U.S. Patent No. 5,629,154; see also, PCT publication WO 97/15687, PCT publication WO 95/13381; Krupp et al. *Nucleic Acids Res.*, 1997, 25: 919; and Wright et al., 1995, *Nuc. Acids Res.* 23:3794, each of which is incorporated herein in its entirety and for all purposes. The dot blot assay is described in detail in co-pending U.S. Patent Application Serial No: 08/833,377, filed April 4, 1997, which is incorporated herein in its entirety and for all purposes. The dot blot assay can be used in a format in which a non-processive activity does not add the 3 or more repeats required for stable hybridization of the (CCC<sub>n</sub>UAA)<sub>n</sub> probe used to detect the activity. Other assays for processive telomerase catalytic activity can also be used, e.g., the stretch PCR assay of Tatematsu et al., 1996, *Oncogene* 13:2265. The gel-blot assay, a combination of the conventional and dot blot assays can also be used. In this variation a conventional assay is performed with no radiolabeled nucleotide and with high dGTP concentrations (e.g., 0.1-2 mM). After performing the conventional assay, the synthesized DNA is separated by denaturing PAGE and transferred to a membrane (e.g., nitrocellulose). Telomeric DNA (the product of telomerase - an extended telomerase primer or substrate) can then be detected by methods such as hybridization using labeled telomeric DNA probes (e.g., probes containing the CCCTAA sequence, as used in the dot blot assay, *supra*) An advantage of this techniques is that it is more sensitive than the conventional assay and provides information about the size of the synthesized fragments and processivity of the reaction.

## c) Activity determinations

The telomerase activity of an hTERT polypeptide can be determined using a unpurified, partially purified or substantially purified hTERT polypeptide (e.g., in association with hTR), *in vitro*, or after expression *in vivo*. For example, telomerase

activity in a cell (e.g., a cell expressing a recombinant hTRT polypeptide of the invention) can be assayed by detecting an increase or decrease in the length of telomeres. Typically assays for telomerase catalytic activity are carried out using an hTRT complexed with hTR; however, alternative telomerase template RNAs may be substituted or one may conduct assays to measure an activity such as telomerase binding. Assays to determine the length of telomeres are known in the art and include hybridization of probes to telomeric DNA (an amplification step can be included) and TRF analysis i.e., the analysis of telomeric DNA restriction fragments [TRFs] following restriction endonuclease digestion, see PCT publications WO 93/23572 and WO 96/41016; Counter et al., 1992, *EMBO J.* 11:1921; Allsopp et al., 1992, *Proc. Nat'l. Acad. Sci. USA* 89:10114; Sanno, 1996, *Am J Clin Pathol* 106:16 and Sanno, 1997, *Neuroendocrinology* 65:299.

The telomerase catalytic activity of a hTRT polypeptide may be determined in a number of ways using the assays *supra* and other telomerase catalytic activity assays. According to one method, the hTRT protein is expressed (e.g., as described *infra*) in a telomerase negative human cell in which hTR is expressed (i.e., either normally in the cell or through recombinant expression), and the presence or absence of telomerase activity in the cell or cell lysate is determined. Examples of suitable telomerase-negative cells are IMR 90 (ATCC, #CCL-186) or BJ cells (human foreskin fibroblast line; see, e.g., Feng et al., 1995, *Science* 269:1236). Other examples include retinal pigmented epithelial cells (RPE), human umbilical vein endothelial cells (HUVEC; ATCC #CRL-1730), human aortic endothelial cells (HAEC; Clonetics Corp, #CC-2535), and human mammary epithelial cells (HME; Hammond et al., 1984, *Proc. Nat'l. Acad. Sci. USA* 81:5435; Stampfer, 1985, *J. Tissue Culture Methods* 9:107). In an alternative embodiment, the hTRT polypeptide is expressed (e.g., by transfection with an hTRT expression vector) in a telomerase positive cell, and an increase in telomerase activity in the cell compared to an untransfected control cell is detected if the polypeptide has telomerase catalytic activity. Usually the telomerase catalytic activity in a cell transfected with a suitable expression vector expressing hTRT will be significantly increased, such as at least about 2-fold, at least about 5-fold, or

even at least about 10-fold to 100-fold or even 1000-fold higher than in untransfected (control) cells.

In an alternative embodiment, the hTRT protein is expressed in a cell (e.g., a telomerase negative cell in which hTR is expressed) as a fusion protein (see 5 *infra*) having a label or an "epitope tag" to aid in purification. In one embodiment, the RNP is recovered from the cell using an antibody that specifically recognizes the tag. Preferred tags are typically short or small and may include a cleavage site or other property that allows the tag to be removed from the hTRT polypeptide. Examples of suitable tags include the Xpress epitope (Invitrogen, Inc., San Diego CA), and other 10 moieties that can be specifically bound by an antibody or nucleic or other equivalent method such as those described in **Example 6**. Alternative tags include those encoded by sequences inserted, e.g., into **SEQ. ID NO: 1** upstream of the ATG codon that initiates translation of the protein of **SEQ. ID. NO: 2**, which may include insertion of a (new) methionine initiation codon into the upstream sequence.

15 It will be appreciated that when a hTRT variant is expressed in a cell (e.g., as a fusion protein) and subsequently isolated (e.g., as a ribonucleoprotein complex), other cell proteins (i.e., telomerase-associated proteins) may be associated with (directly or indirectly bound to) the isolated complex. In such cases, it will sometimes be desirable to assay telomerase activity for the complex containing hTRT, 20 hTR and the associated proteins.

## 2) OTHER TELOMERASE OR TRT PROTEIN ACTIVITIES

The hTRT polypeptides of the invention include variants that lack telomerase catalytic activity but retain one or more other activities of telomerase. 25 These other activities and the methods of the invention for measuring such activities include (but are not limited to) those discussed in the following sections:

### a) Conventional reverse transcriptase activity

Telomerase conventional reverse transcriptase activity is described in, e.g., Morin, 1997, *supra*, and Spence et al., 1995, *Science* 267:988. Because hTRT 30 contains conserved amino acid motifs that are required for reverse transcriptase

catalytic activity, hTERT has the ability to transcribe exogenous RNAs. A conventional RT assay measures the ability of the enzyme to transcribe an RNA template by extending an annealed DNA primer. Reverse transcriptase activity can be measured in numerous ways known in the art, for example monitoring the size increase of a labeled DNA primer, or incorporation of a labeled dNTP. See, e.g., Ausubel et al., *supra*.

Because hTERT specifically associates with hTR, it can be appreciated that the DNA primer/RNA template for a conventional RT assay can be modified to have characteristics related to hTR and a telomeric DNA primer. For example, the RNA can have the sequence (CCCTAA)<sub>n</sub>, where n is at least 1, or at least 3, or at least 10. Thus in one embodiment, the (CCCTAA)<sub>n</sub> region is at or near the 5' terminus of the RNA (similar to the 5' locations of template regions in telomerase RNAs). Similarly, the DNA primer may have a 3' terminus that contains portions of the TTAGGG telomere sequence, for example X<sub>n</sub>TTAG, X<sub>n</sub>AGGG, X<sub>n</sub>(TTAGGG)<sub>q</sub>TTAG, etc., where X is a non-telomeric sequence and n is 8-20, or 6-30, and q is 1-4. In another embodiment, the DNA primer has a 5' terminus that is non-complementary to the RNA template, such that when annealed the 5' terminus remains unbound. Additional modifications of standard reverse transcription assays that may be applied to the methods of the invention are known in the art.

#### b) Nucleolytic activity

Telomerase nucleolytic activity is described in e.g., Morin, 1997, *supra*; Collins and Grieder, 1993, *Genes and Development* 7:1364. Telomerase possesses a nucleolytic activity (Joyce and Steitz, 1987, *Trends Biochem. Sci.* 12:288), however the telomerase activity has defining characteristics. Telomerase preferentially removes nucleotides, usually only one, from the 3' end when the 3' end of the DNA is positioned at the 5' boundary of the DNA template, in humans and *Tetrahymena* this nucleotide is the first G of the TTAGG repeat. Telomerase preferentially removes G residues but has nucleolytic activity against other nucleotides. This activity can be monitored. Two different methods are described here for illustrative purposes. One method involves a conventional telomerase reaction with a primer that binds the entire template sequence (i.e., terminating at the template boundary; -TAGGGATTAG in humans). Nucleolytic

activity is observed by monitoring the replacement of the last dG residue with a radiolabeled dGTP provided in the assay. The replacement is monitored by the appearance of a band at the size of the starting primer as shown by gel electrophoresis and autoradiography.

5           A preferred method uses a DNA primer that has a "blocked" 3' terminus that cannot be extended by telomerase. The 3' blocked primer can be used in a standard telomerase assays but will not be extended unless the 3' nucleotide is removed by the nucleolytic activity of telomerase. The advantage of this method is that telomerase activity can be monitored by any of several standard means and the signal is strong and  
10       easy to quantify. The blocking of the 3' terminus of the primer can be accomplished in several ways. One method is the addition of a 3'-deoxy-dNTP residue at the 3' terminus of the primer using standard oligonucleotide synthesis techniques. This terminus has a 2' OH but not the 3' OH required for telomerase. Other means of blocking the 3' terminus exist, for instance, a 3' dideoxy terminus, a 3'-amine terminus, and others. An  
15       example of a primer for an hTRT nucleolytic assay is 5' -TTAGGGTTAGGGTTA (G<sub>3'H</sub>) where the latter residue denotes a 3'-deoxy-guanosine residue (Glen Research, Sterling, VA). Numerous other variations for a suitable primer based on the disclosure are known to those of skill in the art.

c) Primer (telomere) binding activity

20           Telomerase primer (telomere) binding activity is described in e.g., Morin, 1997, *supra*; Collins et al., 1995, *Cell* 81:677; Harrington et al, 1995, *J. Biol. Chem.* 270:8893. Telomerase is believed to have two sites which bind a telomeric DNA primer. The RT motifs associated with primer binding indicates hTRT and/or hTRT/hTR possesses DNA primer binding activity. There are several ways of assaying  
25       primer binding activity; however, a step common to most methods is incubation of a labeled DNA primer with hTRT or hTRT/hTR or other TRT/TR combinations under appropriate binding conditions. Also, most methods employ a means of separating unbound DNA from protein-bound DNA, those methods include the following.

          i) Gel-shift assays (also called electrophoretic/mobility shift assays) are  
30       those in which unbound DNA primer is separated from protein-bound DNA primer by

electrophoresis on a nondenaturing gel (Ausubel et al., *supra*).

ii) Matrix binding assays include several variations to the basic technique, which involves binding the hTRT or hTRT/hTR complex to a matrix (e.g., nitrocellulose), either before or after incubation with the labeled primer. By binding the hTRT to a matrix, the unbound primer can be mechanically separated from bound primer. Residual unbound DNA can be removed by washing of the membrane prior to quantitation. Those of skill recognize there are several means of coupling proteins to such matrices, solid supports, and membranes, including chemical, photochemical, UV crosslinking, antibody/epitope, and non-covalent (hydrophobic, electrostatic, etc.) interactions.

The DNA primer may be any DNA with an affinity for telomerase, such as, for example, a telomeric DNA primer like (TTAGGG)<sub>n</sub> where n could be 1-10 and is typically 3-5. The 3' and 5' termini could end in any location of the repeat sequence. The primer may also have 5' or 3' extensions of non-telomeric DNA that could facilitate labeling or detection. The primer may also be derivatized, e.g., to facilitate detection or isolation.

d) dNTP binding activity

Telomerase dNTP binding activity is described in e.g., Morin, 1997, *supra*; Spence et al., *supra*. Telomerase requires dNTPs to synthesize DNA. The hTRT protein has a nucleotide binding activity and can be assayed for dNTP binding in a manner similar to other nucleotide binding proteins (Kantrowitz et al., 1980, *Trends Biochem. Sci.* 5:124). Typically, binding of a labeled dNTP or dNTP analog is monitored, as is known in the art for non-telomerase RT proteins..

e) RNA (i.e., hTR) binding activity

Telomerase RNA (i.e., hTR) binding activity is described in e.g., Morin, 1997, *supra*; Harrington et al., 1997, *Science* 275:973; Collins et al., 1995, *Cell* 81:677. The RNA binding activity of a TRT protein of the invention may be assayed in a manner similar to the DNA primer binding assay described *supra*, using a labeled RNA probe. Methods for separating bound and unbound RNA, and for detecting RNA are well known in the art and can be applied to the activity assays of the invention in a

manner similar to that described for the DNA primer binding assay. The RNA can be full length hTR, fragments of hTR or other RNAs sequences demonstrated to have an affinity for telomerase or hTRT. See U.S. Patent No. 5,583,016 and PCT Pub. No. 96/40868 (see also USSN 08/478,352, filed 7 June 1995).

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### 3) TELOMERASE MOTIFS AS TARGETS

The present invention, as noted *supra*, provides hTRT polypeptides having less than the full complement (as described *supra*) of the telomerase activities of naturally occurring telomerase or hTRT or other TRT proteins. It will be appreciated that, in view of the disclosure herein of the RT and telomerase-specific motifs of TRT, that alteration or mutation of conserved amino acid residues, such as are found in the motif sequences discussed *supra*, will result in loss-of activity mutants useful for therapeutic, drug screening and characterization, and other uses. For example, as described in **Example 1**, deletion of motifs B through D in the RT domains of the endogenous TRT gene in *S. pombe* resulted in haploid cells in which progressive telomere shortening to the point where hybridization to telomeric repeats became almost undetectable was observed, indicating a loss of telomerase catalytic activity. Similarly, alterations in the WxGxS site of motif E can affect telomerase DNA primer binding or function. Additionally, alterations of the amino acids in the motifs A, B', and C can affect the catalytic activity of telomerase. Mutation of the DD motif of hTRT can significantly reduce or abolish telomerase activity (see Example 16).

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### C) SYNTHESIS OF HTRT AND OTHER TRT POLYPEPTIDES

The invention provides a variety of methods for making the hTRT and other TRT polypeptides disclosed herein. In the following sections, chemical synthesis and recombinant expression of hTRT proteins, including fusion proteins, is described in some detail.

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#### 1) CHEMICAL SYNTHESIS

The invention provides hTRT polypeptides synthesized, entirely or in part, using general chemical methods well known in the art (see e.g., Caruthers et al.,

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1980, *Nucleic Acids Res. Symp. Ser.*, 215-223; and Horn et al., 1980, *Nucleic Acids Res. Symp. Ser.*, 225-232). For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, et al., 1995, *Science* 269:202) including automated synthesis (e.g., using the Perkin Elmer ABI 431A Peptide Synthesizer in accordance with the instructions provided by the manufacturer). When full length protein is desired, shorter polypeptides may be fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule to form a peptide bond.

The newly synthesized peptide can be substantially purified, for example, by preparative high performance liquid chromatography (e.g., Creighton, PROTEINS, STRUCTURES AND MOLECULAR PRINCIPLES, WH Freeman and Co, New York NY [1983]). The composition of the synthetic peptides (or any other peptides or polypeptides of the invention) may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*). Importantly, the amino acid sequence of hTRT, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins or otherwise, or any part thereof or for any purpose, to produce a variant polypeptide of the invention.

## 2) RECOMBINANT EXPRESSION OF hTRT AND OTHER TRT PROTEINS

The present invention provides methods, reagents, vectors, and cells useful for expression of hTRT polypeptides and nucleic acids using *in vitro* (cell-free), *ex vivo* or *in vivo* (cell or organism-based) recombinant expression systems. In one embodiment, expression of the hTRT protein, or fragment thereof, comprises inserting the coding sequence into an appropriate expression vector (*i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted coding sequence). Thus, in one aspect, the invention provides for a polynucleotide substantially identical in sequence to an hTRT gene coding sequence at least 25 nucleotides, and preferably for many applications 50 to 100 nucleotides or more, of the hTRT cDNAs or genes of the invention, which is operably linked to a promoter to form



a transcription unit capable of expressing an hTRT polypeptide. Methods well known to those skilled in the art can be used to construct the expression vectors containing an hTRT sequence and appropriate transcriptional or translational controls provided by the present invention (see, e.g., Sambrook et al., *supra*, Ausubel et al. *supra*, and this disclosure).

The hTRT polypeptides provided by the invention include fusion proteins that contain hTRT polypeptides or fragments of the hTRT protein. The fusion proteins are typically produced by recombinant means, although they may also be made by chemical synthesis. Fusion proteins may be useful in providing enhanced expression of the hTRT polypeptide constructs, or in producing hTRT polypeptides having other desirable properties, for example, comprising a label (such as an enzymatic reporter group), binding group, or antibody epitope. An exemplary fusion protein, comprising hTRT and enhanced green fluorescent protein (EGFP) sequences is described in **Example 15**, *infra*. It will be apparent to one of skill that the uses and applications discussed in **Example 15** and elsewhere herein are not limited to the particular fusion protein, but are illustrative of the uses of various fusion constructs.

The fusion protein systems of the invention can also be used to facilitate efficient production and isolation of hTRT proteins or peptides. For example, in some embodiments, the non-hTRT sequence portion of the fusion protein comprises a short peptide that can be specifically bound to an immobilized molecule such that the fusion protein can be separated from unbound components (such as unrelated proteins in a cell lysate). One example is a peptide sequence that is bound by a specific antibody. Another example is a peptide comprising polyhistidine tracts e.g. (His)<sub>6</sub> or histidine-tryptophan sequences that can be bound by a resin containing nickel or copper ions (i.e., metal-chelate affinity chromatography). Other examples include Protein A domains or fragments, which allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle WA). In some embodiments, the fusion protein includes a cleavage site so that the hTRT or other TRT polypeptide sequence can be easily separated from the leader sequence. In this case, cleavage may be chemical (e.g., cyanogen bromide, 2-(2-

nitrophenylsulphenyl)-3-methyl-3'-bromoindolene, hydroxylamine, or low pH) or enzymatic (e.g., Factor Xa, enterokinase). The choice of the fusion and cleavage systems may depend, in part, on the portion (i.e., sequence) of the hTERT polypeptide being expressed. Fusion proteins generally are described in Ausubel et al., *supra*, Ch. 5 16, Kroll et al., 1993, *DNA Cell. Biol.* 12:441, and the Invitrogen 1997 Catalog (Invitrogen Inc, San Diego CA). Other exemplary fusion proteins of the invention with epitope tags or tags and cleavage sites are provided in **Example 6, *infra***.

It will be appreciated by those of skill that, although the expression systems discussed in this section are focused on expression of hTERT polypeptides, the same or similar cells, vectors and methods may be used to express hTERT 10 polynucleotides of the invention, including sense and antisense polynucleotides without necessarily deserving to produce hTERT polypeptides. Typically, expression of a polypeptide requires a suitable initiation codon (e.g., methionine), open reading frame, and translational regulatory signals (e.g., a ribosome binding site, a termination codon) 15 which may be omitted when translation of a nucleic acid sequence to produce a protein is not desired.

Expression of hTERT polypeptides and polynucleotides may be carried out to accomplish any of several related benefits provided by the present invention. One illustrative benefit is expression of hTERT polypeptides that are subsequently 20 isolated from the cell in which they are expressed (for example for production of large amounts of hTERT for use as a vaccine). A second illustrative benefit is expression of hTERT in a cell to change the phenotype of the cell (as in gene therapy applications). Nonmammalian cells can be used for expression of hTERT for purification, while eukaryotic especially mammalian cells (e.g., human cells) can be used not only for 25 isolation and purification of hTERT but also for expression of hTERT when a change in proliferative capacity in a cell is desired (e.g., to effect a change in phenotype as in gene therapy applications). By way of illustration and not limitation, hTERT polypeptides having one or more telomerase activities (e.g. telomerase catalytic activity) can be expressed in a host cell to increase the proliferative capacity of a cell (e.g., immortalize 30 a cell) and, conversely, hTERT antisense polynucleotides or inhibitory polypeptides

typically can be expressed to reduce the proliferative capacity of a cell (e.g., of a telomerase positive malignant tumor cell). Numerous specific applications are described herein, e.g., in the discussion of uses of the reagents and methods of the invention for therapeutic applications, below.

5 Illustrative useful expression systems (cells, regulatory elements, vectors and expression) of the present invention include a number of cell-free systems such as reticulocyte lysate and wheat germ systems using hTRT polynucleotides in accordance with general methods well known in the art (see, e.g., Ausubel et al. *supra* at Ch. 10). In alternative embodiments, the invention provides reagents and methods for expressing  
10 hTRT in prokaryotic or eukaryotic cells. Thus, the present invention provides nucleic acids encoding hTRT polynucleotides, proteins, protein subsequences, or fusion proteins that can be expressed in bacteria, fungi, plant, insect, and animal, including human, cell expression systems known in the art, including isolated cells, cell lines, cell cultures, tissues, and whole organisms. As will be understood by those of skill, the  
15 hTRT polynucleotides introduced into a host cell or cell free expression system will usually be operably linked to appropriate expression control sequences for each host or cell free system.

Useful bacterial expression systems include *E. coli*, bacilli (such as *Bacillus subtilis*), other enterobacteriaceae (such as *Salmonella*, *Serratia*, and various  
20 *Pseudomonas species*) or other bacterial hosts (e.g., *Streptococcus cremoris*, *Streptococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc citrovorum*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacteriu breve*, and *Bifidobacterium longum*). The hTRT expression constructs useful in prokaryotes include recombinant bacteriophage,  
25 plasmid or cosmid DNA expression vectors, or the like, and typically include promoter sequences. Illustrative promoters include inducible promoters, such as the *lac* promoter, the hybrid *lacZ* promoter of the Bluescript7 phagemid [Stratagene, La Jolla CA] or pSport1 [Gibco BRL]; phage lambda promoter systems; a tryptophan (*trp*) promoter system; and *ptrp-lac* hybrids and the like. Bacterial expression constructs  
30 optionally include a ribosome binding site and transcription termination signal

regulatory sequences. Illustrative examples of specific vectors useful for expression include, for example, pTrcHis2, (Invitrogen, San Diego CA), and numerous others known in the art or that may be developed (see, e.g. Ausubel). Useful vectors for bacteria include those that facilitate production of hTERT- fusion proteins. Useful  
5 vectors for high level expression of fusion proteins in bacterial cells include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as Bluescript7 (Stratagene), noted above, in which the sequence encoding hTERT protein, an hTERT fusion protein or an hTERT fragment may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of  
10  $\beta$ -galactosidase so that a hybrid protein is produced (e.g., pIN vectors; Van Heeke and Schuster, 1989, *J. Biol. Chem.*, 264:5503). Vectors such as pGEX vectors (e.g., pGEX-2TK; Pharmacia Biotech) may also be used to express foreign polypeptides, such as hTERT protein, as fusion proteins with glutathione S-transferase (GST). Such fusion proteins may be purified from lysed cells by adsorption to glutathione-agarose beads  
15 followed by elution in the presence of free glutathione. Proteins made in such systems often include enterokinase, thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will, as may be useful in purification or other applications. Other examples are fusion proteins comprising hTERT and the *E. coli* Maltose Binding Protein (MBP) or *E. Coli*  
20 thioredoxin. Illustrative examples of hTERT expression constructs useful in bacterial cells are provided in **Example 6, infra**.

The invention further provides hTERT polypeptides expressed in fungal systems, such as dictyostelium and, preferably, yeast, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Torulopsis holmil*, *Saccharomyces fragilis*, *Saccharomyces*  
25 *lactis*, *Hansenula polymorpha* and *Candida pseudotropicalis*. When hTERT is expressed in yeast, a number of suitable vectors are available, including plasmid and yeast artificial chromosomes (YACs) vectors. The vectors typically include expression control sequences, such as constitutive or inducible promoters (e.g., such as alpha factor, alcohol oxidase, PGH, and 3-phosphoglycerate kinase or other glycolytic  
30 enzymes), and an origin of replication, termination sequences and the like, as desired.

Suitable vectors for use in *Pichia* include pPICZ, His6/pPICZB, pPICZalpha, pPIC3.5K, pPIC9K, pA0815, pGAP2A, B & C, pGAP2alpha A, B, and C (Invitrogen, San Diego, CA) and numerous others known in the art or to be developed. In one embodiment, the vector His6/pPICZB (Invitrogen, San Diego, CA) is used to express a His<sub>6</sub>-hTRT fusion protein in the yeast *Pichia pastoris*. An example of a vector useful in *Saccharomyces* is pYES2 (Invitrogen, San Diego, CA). Illustrative examples of hTRT expression constructs useful in yeast are provided in **Example 6, infra**.

The hTRT polypeptides of the invention may also be expressed in plant cell systems transfected with plant or plant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (e.g., Ti or pBR322 plasmid). In cases where plant virus expression vectors are used, the expression of an hTRT-encoding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV (Brisson et al., 1984, *Nature* 310:511-514) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu et al., 1987, *EMBO J.*, 6:307-311). Alternatively, plant promoters such as that from the small subunit gene of RUBISCO (Coruzzi et al., 1984, *EMBO J.*, 3:1671-1680; Broglie et al., 1984, *Science* 224:838-843) or heat shock promoters (Winter and Sinibaldi, 1991, *Results Probl. Cell Differ.*, 17:85), or storage protein gene promoters may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection (for reviews of such techniques, see Hobbs or Murry, 1992, in MCGRAW HILL YEARBOOK OF SCIENCE AND TECHNOLOGY McGraw Hill New York NY, pp. 191-196 [1992]; or Weissbach and Weissbach, 1988, METHODS FOR PLANT MOLECULAR BIOLOGY, Academic Press, New York NY, pp. 421-463).

Another expression system provided by the invention for expression of hTRT protein is an insect system. A preferred system uses a baculovirus polyhedrin promoter. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The sequence encoding the gene of interest may be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control

of the polyhedrin promoter. Successful insertion of the sequence, e.g., encoding the hTRT protein, will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses are then used to infect *S. frugiperda* cells or *Trichoplusia* larvae, in which the hTRT sequence is then expressed (see, for general methods, Smith et al., *J. Virol.*, 46:584 [1983]; Engelhard et al., *Proc. Natl. Acad. Sci.* 91:3224-7 [1994]). Useful vectors for baculovirus expression include pBlueBacHis2 A, B & C, pBlueBac4.5, pMelBacB and numerous others known in the art or to be developed. Illustrative examples of hTRT expression constructs useful in insect cells are provided in **Example 6, *infra***.

The present invention also provides expression systems in mammals and mammalian cells. As noted *supra*, hTRT polynucleotides may be expressed in mammalian cells (e.g., human cells) for production of significant quantities of hTRT polypeptides (e.g., for purification) or to change the phenotype of a target cell (e.g., for purposes of gene therapy, cell immortalization, or other). In the latter case, the hTRT polynucleotide expressed may or may not encode a polypeptide with a telomerase catalytic activity. That is, expression may be of a sense or antisense polynucleotide, an inhibitory or stimulatory polypeptide, a polypeptide with zero, one or more telomerase activities, and other combinations and variants disclosed herein or apparent to one of skill upon review of this disclosure.

Suitable mammalian host tissue culture cells for expressing the nucleic acids of the invention include any normal mortal or normal or abnormal immortal animal or human cell, including: monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293) (Graham et al., *J. Gen. Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); CHO (ATCC CCL 61 and CRL 9618); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL 1587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC

CCL51); and, TRI cells (Mather, et al., *Annals N.Y. Acad. Sci.* 383:44-46 (1982); MDCK (ATCC CCL 34 and CRL 6253), HEK 293 (ATCC CRL 1573), WI-38 (ATCC CCL 75) (ATCC: American Type Culture Collection, Rockville, MD)). The use of mammalian tissue cell culture to express polypeptides is discussed generally in Winnacker, FROM GENES TO CLONES (VCH Publishers, N.Y., N.Y., 1987).

For mammalian host cells, viral-based and nonviral expression systems are provided. Nonviral vectors and systems include plasmids, episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (see, e.g., Harrington et al., 1997, *Nat Genet* 15:345), for example, nonviral vectors useful for expression of hTERT polynucleotides and polypeptides in mammalian (e.g., human) cells include pThioHis A, B & C, pcDNA3.1/His, pEBVHis A, B & C, (Invitrogen, San Diego CA), MPSV vectors, others described in the Invitrogen 1997 Catalog (Invitrogen Inc, San Diego CA) which is incorporated in its entirety herein, and numerous others known in the art for other proteins. Illustrative examples of hTERT expression constructs useful in mammalian cells are provided in **Example 6, *infra***.

Useful viral vectors include vectors based on retroviruses, adenoviruses, adenoassociated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). SFV and vaccinia vectors are discussed generally in Ausubel et al., *supra*, Ch 16. These vectors are often made up of two components, a modified viral genome and a coat structure surrounding it (see generally Smith, 1995, *Annu. Rev. Microbiol.* 49: 807), although sometimes viral vectors are introduced in naked form or coated with proteins other than viral proteins. However, the viral nucleic acid in a vector may be changed in many ways, for example when designed for gene therapy. The goals of these changes are to disable growth of the virus in target cells while maintaining its ability to grow in vector form in available packaging or helper cells, to provide space within the viral genome for insertion of exogenous DNA sequences, and to incorporate new sequences that encode and enable appropriate expression of the gene of interest. Thus, vector nucleic acids generally comprise two components: essential cis-acting viral sequences for

replication and packaging in a helper line and the transcription unit for the exogenous gene. Other viral functions are expressed in trans in a specific packaging or helper cell line. Adenoviral vectors (e.g., for use in human gene therapy) are described in, e.g., Rosenfeld et al., 1992, *Cell* 68: 143; PCT publications WO 94/12650; 94/12649; and 94/12629. In cases where an adenovirus is used as an expression vector, a sequence encoding hTERT may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome will result in a viable virus capable of expressing in infected host cells (Logan and Shenk, 1984, *Proc. Natl. Acad. Sci.*, 81:3655). Replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome are described in, e.g., Miller et al., 1990, *Mol. Cell. Biol.* 10: 4239; Kolberg, 1992, *J. NIH Res.* 4: 43; and Cornetta et al., 1991, *Hum. Gene Ther.* 2: 215.

In mammalian cell systems, promoters from the mammalian genes or from mammalian viruses are often appropriate. Suitable promoters may be constitutive, cell type-specific, stage-specific, and/or modulatable or regulatable (e.g., by hormones such as glucocorticoids). Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art.

Other regulatory elements may also be required or desired for efficient expression of an hTERT polynucleotide and/or translation of a sequence encoding hTERT proteins. For translation, these elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. For sequences encoding the hTERT protein, provided its initiation codon and upstream promoter sequences are inserted into an expression vector, no additional translational or other control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous transcriptional and/or translational control signals (e.g., the promoter,



ribosome-binding site, and ATG initiation codon) must often be provided.

Furthermore, the initiation codon must typically be in the correct reading frame to ensure translation of the desired protein. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf et al., 1994, *Results Probl. Cell Differ.* 20:125; and Bittner et al. 1987, *Meth. Enzymol.*, 153:516). For example, the SV40 enhancer or CMV enhancer may be used to increase expression in mammalian host cells.

Expression of hTERT gene products can also be effected (increased) by activation of an hTERT promoter or enhancer in a cell such as a human cell, e.g., a telomerase-negative cell line. Activation can be carried out in a variety of ways, including administration of an exogenous promoter activating agent, or inhibition of a cellular component that suppresses expression of the hTERT gene. It will be appreciated that, conversely, inhibition of promoter function, as described *infra*, will reduce hTERT gene expression.

The invention provides inducible and repressible expression of hTERT polypeptides using such system as the Ecdysone-Inducible Expression System (Invitrogen), and the Tet-On and Tet-off tetracycline regulated systems from Clontech. The ecdysone-inducible expression system uses the steroid hormone ecdysone analog, muristerone A, to activate expression of a recombinant protein via a heterodimeric nuclear receptor (No et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:3346). In one embodiment of the invention, hTERT is cloned in the pIND vector (Clontech), which contains 5 modified ecdysone response elements (E/GREs) upstream of a minimal heat shock promoter and the multiple cloning site. The construct is then transfected in cell lines stably expressing the ecdysone receptor. After transfection, cells are treated with muristerone A to induce intracellular expression from pIND. In another embodiment of the invention, hTERT polypeptide is expressed using the Tet-on and Tet-off expression systems (Clontech) provide to the regulated, high-level gene expression systems described (Gossen et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:5547; Gossen et al., 1995, *Science* 268:1766).

## PATENT

The hTERT vectors of the invention may be introduced into a cell, tissue, organ, patient or animal by a variety of methods. The nucleic acid expression vectors (typically dsDNA) of the invention can be transferred into the chosen host cell by well-known methods such as calcium chloride transformation (for bacterial systems),  
5 electroporation, calcium phosphate treatment, liposome-mediated transformation, injection and microinjection, ballistic methods, virosomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA, artificial virions, fusion to the herpes virus structural protein VP22 (Elliot and O'Hare, *Cell* 88:223), agent-enhanced uptake of DNA, and *ex vivo* transduction. Useful liposome-mediated DNA transfer methods  
10 are described in US Patent Nos. 5,049,386, US 4,946,787; and US 4,897,355; PCT publications WO 91/17424, WO 91/16024; Wang and Huang, 1987, *Biochem. Biophys. Res. Commun.* 147: 980; Wang and Huang, 1989, *Biochemistry* 28: 9508; Litzinger and Huang, 1992, *Biochem. Biophys. Acta* 1113:201; Gao and Huang, 1991, *Biochem. Biophys. Res. Commun.* 179: 280. Immunoliposomes have been described as carriers  
15 of exogenous polynucleotides (Wang and Huang, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:7851; Trubetskoy et al., 1992, *Biochem. Biophys. Acta* 1131:311 and may have improved cell type specificity as compared to liposomes by virtue of the inclusion of specific antibodies which presumably bind to surface antigens on specific cell types. Behr et al., 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6982 report using lipopolyamine as a  
20 reagent to mediate transfection itself, without the necessity of any additional phospholipid to form liposomes. Suitable delivery methods will be selected by practitioners in view of acceptable practices and regulatory requirements (e.g., for gene therapy or production of cell lines for expression of recombinant proteins). It will be appreciated that the delivery methods listed above may be used for transfer of nucleic  
25 acids into cells for purposes of gene therapy, transfer into tissue culture cells, and the like.

For long-term, high-yield production of recombinant proteins, stable expression will often be desired. For example, cell lines which stably express hTERT can be prepared using expression vectors of the invention which contain viral origins of  
30 replication or endogenous expression elements and a selectable marker gene.

Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth of cells which successfully express the introduced sequences in selective media.

- 5     Resistant, stably transfected cells can be proliferated using tissue culture techniques appropriate to the cell type. An amplification step, e.g., by administration of methyltrexate to cells transfected with a DHFR gene according to methods well known in the art, can be included.

- 10             In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, phosphorylation, lipidation and acylation. Post-translational processing may also be important for correct insertion, folding and/or function. Different host cells have cellular machinery and characteristic mechanisms specific for
- 15     each cell for such post-translational activities and so a particular cell may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

- The present invention also provides transgenic animals (i.e., mammals transgenic for a human or other TRT gene sequence) expressing an hTRT or other TRT polynucleotide or polypeptide. In one embodiment, hTRT is secreted into the milk of a
- 20     transgenic mammal such as a transgenic bovine, goat, or rabbit. Methods for production of such animals are found, e.g., in Heyneker et al., PCT WO 91/08216.

- The hTRT proteins and complexes of the invention, including those made using the expression systems disclosed herein *supra*, may be purified using a variety of general methods known in the art in accordance with the specific methods
- 25     provided by the present invention (e.g., *infra*). One of skill in the art will recognize that after chemical synthesis, biological expression, or purification, the hTRT protein may possess a conformation different than a native conformation of naturally occurring telomerase. In some instances, it may be helpful or even necessary to denature (e.g., including reduction of disulfide or other linkages) the polypeptide and then to cause the
- 30     polypeptide to re-fold into the preferred conformation. Productive refolding may also

require the presence of hTR (or hTR fragments). Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (see, e.g., Debinski et al., 1993, *J. Biol. Chem.*, 268:14065; Kreitman and Pastan, 1993, *Bioconjug. Chem.*, 4:581; and Buchner et al., 1992, *Anal. Biochem.*, 205:263; and  
5 McCaman et al., 1985, *J. Biotech.* 2:177). See also USSN 08/478,352, filed 7 June 1995, *supra*.

**D) COMPLEXES OF HUMAN TRT AND HUMAN TELOMERASE  
RNA, TELOMERASE-ASSOCIATED PROTEINS, AND OTHER  
10 BIOMOLECULES PRODUCED BY COEXPRESSION AND OTHER MEANS**

hTRT polypeptides of the invention can associate *in vivo* and *in vitro* with other biomolecules, including RNAs (e.g., hTR), proteins (e.g., telomerase-associated proteins), DNA (e.g., telomeric DNA,  $[T_2AG_3]_N$ ), and nucleotides, such as (deoxy)ribonucleotide triphosphates. These associations can be exploited to assay  
15 hTRT presence or function, to identify or purify hTRT or telomerase-associated molecules, and to analyze hTRT or telomerase structure or function in accordance with the methods of the present invention.

In one embodiment, the present invention provides hTRT complexed with (e.g., associated with or bound to) a nucleic acid, usually an RNA. In one  
20 embodiment, the bound RNA is capable of acting as a template for telomerase-mediated DNA synthesis. Examples of RNAs that may be complexed with the hTRT polypeptide include a naturally occurring host cell telomerase RNA, a human telomerase RNA (e.g., hTR; U.S. Patent No. 5,583,016), an hTR subsequence or domain, a synthetic RNA, or other RNAs. The RNA-hTRT protein complex (an RNP)  
25 typically exhibit one or more telomerase activities, such as telomerase catalytic activities. These hTRT-hTR RNPs (or other hTRT-RNA complexes) can be produced as a variety of methods, as described *infra* for illustrative purposes, including *in vitro* reconstitution, and co-expression of hTRT and hTR (or other RNA) *in vitro* (i.e., in a cell free system), *in vivo*, or *ex vivo*.

30 Thus, the present invention provides, in one embodiment, an hTRT-hTR

complex (or other hTRT-RNA complex) formed *in vitro* by mixing separately purified components (“*in vitro* reconstitution;” see, e.g., U.S. Patent No. 5,583,016 for a description of reconstitution and USSN 08/478,352, filed 7 June 1995; also see Autexier et al., *EMBO J.* 15:5928).

5                   In an alternative embodiment, the invention provides telomerase RNPs produced by coexpression of the hTRT polypeptide and an RNA (e.g., hTR) *in vitro* in a cell-free transcription-translation system (e.g. wheat germ or rabbit reticulocyte lysate). As shown in **Example 7**, *in vitro* co-expression a recombinant hTRT polypeptide and hTR results in production of telomerase catalytic activity (as measured  
10                   by a TRAP assay).

                  Further provided by the present invention are telomerase RNPs produced by expression of the hTRT polypeptide in a cell, e.g., a mammalian cell, in which hTR is naturally expressed or in which hTR (or another RNA capable of forming a complex with the hTRT protein) is introduced or expressed by recombinant means. Thus, in one  
15                   embodiment, hTRT is expressed in a telomerase negative human cell in which hTR is present (e.g., BJ or IMP90 cells), allowing the two molecules to assemble into an RNP. In another embodiment, hTRT is expressed in a human or non-human cell in which hTR is recombinantly expressed. Methods for expression of hTR in a cell are found in U.S. Patent 5,583,016. Further, a clone containing a cDNA encoding the RNA  
20                   component of telomerase has been placed on deposit as pGRN33 (ATCC 75926). Genomic sequences encoding the RNA component of human telomerase are also on deposit in the ~15 kb *Sau*III A1 to *Hind*III insert of clone 28-1 (ATCC 75925). For expression in eukaryotic cells the hTRT sequence will typically be operably linked to a transcription initiation sequence (RNA polymerase binding site) and transcription  
25                   terminator sequences (see, e.g., PCT Publication WO 96/01835; Feng et al., 1995, *Science* 269:1236).

                  The present invention further provides recombinantly produced or substantially purified hTRT polypeptides coexpressed and/or associated with so-called “telomerase-associated proteins.” Thus, the present invention provides hTRT  
30                   coexpressed with, or complexed with, other proteins (e.g., telomerase-associated

proteins). Telomerase-associated proteins are those proteins that copurify with human telomerase and that may play a role in modulating telomerase function or activity, for example by participating in the association of telomerase with telomeric DNA.

Examples of telomerase-associated proteins include (but are not limited to) the following proteins and/or their human homologs: nucleolin (*see*, copending U.S. patent application serial no. 08/833,377, and Srivastava et al., 1989, *FEBS Letts.* 250:99); EF2H (elongation factor 2 homolog; *see*, copending U.S. patent application no. 08/833,377 and Nomura et al. 1994, *DNA Res. (Japan)* 1:27, GENBANK accession #D21163); TP1 (Harrington et al., 1997, *Science* 275:973; the human homologue of the *Tetrahymena* p95 (Collins et al., 1995, *Cell* 81:677); TPC2 (a telomere length regulatory protein; ATCC accession number 97708 (see USSN 08/710,249 and 08/713,922 both filed 13 September 1996); TPC3 (also a telomere length regulatory protein; ATCC accession number 97707 (see USSN 08/710,249 and 08/713,922 both filed 13 September 1996); DNA-binding protein B (dbpB; Horwitz et al., 1994, *J. Biol. Chem.* 269:14130; and Telomere Repeat Binding Factor (TRF 1 & 2; Chang et al., 1995, *Science* 270:1663; Chong et al., 1997, *Hum Mol Genet* 6:69); EST1, 3 and 4 (Lendvay et al., 1996, *Genetics* 144:1399, Nugent et al., 1996, *Science* 274:249 Lundblad et al., 1989, *Cell* 57:633); and End-capping factor (Cardenas et al., 1993, *Genes Dev.* 7:883).

Telomerase associated proteins can be identified on the basis of co-purification with, or binding to, hTERT protein or the hTERT-hTR RNP. Alternatively, they can be identified on the basis of binding to an hTERT fusion protein, e.g., a GST-hTERT fusion protein or the like, as determined by affinity purification (*see*, Ausubel et al. Ch 20). A particularly useful technique for assessing protein-protein interactions and identifying hTERT-associated proteins is the two hybrid screen method of Chien et al. (*Proc. Natl. Acad. Sci. USA* 88:9578 [1991]; *see also* Ausubel et al., *supra*, at Ch. 20). This screen identifies protein-protein interactions *in vivo* through reconstitution of a transcriptional activator, the yeast Gal4 transcription protein (*see*, Fields and Song, 1989, *Nature* 340:245. The method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional

activation. Polynucleotides, usually expression vectors, encoding two hybrid proteins are constructed. One polynucleotide comprises the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a protein to be tested for an hTRT interaction (e.g., nucleolin or EF2H). Alternatively the yeast Gal4 DNA-binding domain is fused to cDNAs from a human cell, thus creating a library of human proteins fused to the Gal4 DNA binding domain for screening for telomerase associated proteins. The other polynucleotide comprises the Gal4 activation domain fused to an hTRT polypeptide sequence. The constructs are introduced into a yeast host cell. Upon expression, intermolecular binding between hTRT and the test protein can reconstitute the Gal4 DNA-binding domain with the Gal4 activation domain. This leads to the transcriptional activation of a reporter gene (e.g., lacZ, HIS3) operably linked to a Gal4 binding site. By selecting for, or assaying the reporter gene colonies of cell that contain an hTRT interacting protein or telomerase associated protein can be identified. Those of skill will appreciate that there are numerous variations of the 2-hybrid screen, e.g., the LexA system (Bartel et al, 1993, *in Cellular Interactions in Development: A Practical Approach* Ed. Hartley, D.A. (Oxford Univ. Press) pp. 153-79).

Another useful method for identifying telomerase-associated proteins is a three-hybrid system (see, e.g., Zhang et al., 1996, *Anal. Biochem.* 242:68; Licitra et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:12817). The telomerase RNA component can be utilized in this system with the TRT or hTRT protein and a test protein. Another useful method for identifying interacting proteins, particularly (i.e., proteins that heterodimerize or form higher order heteromultimers) is the *E. coli*/BCCP interactive screening system (see, Germino et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:933; Guarente (1993) *Proc. Natl. Acad. Sci. (U.S.A.)* 90:1639).

The present invention also provides complexes of telomere binding proteins (which may or may not be telomerase associated proteins) and hTRT (which may or may not be complexed with hTR, other RNAs, or one or more telomerase associated proteins. Examples of telomere binding proteins include TRF1 and TRF2 (*supra*); rnpA1, rnpA2, RAP1 (Buchman et al., 1988, *Mol. Cell. Biol.* 8:210, Buchman et al., 1988, *Mol. Cell. Biol.* 8:5086), SIR3 and SIR4 (Aparicio et al, 1991, *Cell*

66:1279), TEL1 (Greenwell et al., 1995, *Cell* 82:823; Morrow et al., 1995, *Cell* 82:831); ATM (Savitsky et al., 1995, *Science* 268:1749) and corresponding human homologues. The aforementioned complexes may be produced generally as described *supra* for complexes of hTRT and hTR or telomerase associated proteins, e.g., by  
5 mixing or co-expression *in vitro* or *in vivo*.

## V. ANTIBODIES AND OTHER BINDING AGENTS

In a related aspect, the present invention provides antibodies that are specifically immunoreactive with hTRT, including polyclonal and monoclonal  
10 antibodies, antibody fragments, single chain antibodies, human and chimeric antibodies, including antibodies or antibody fragments fused to phage coat or cell surface proteins, and others known in the art and described herein. The antibodies of the invention can specifically recognize and bind polypeptides that have an amino acid sequence that is substantially identical to the amino acid sequence of **SEQ. ID. NO: 2**,  
15 or an immunogenic fragment thereof or epitope on the protein defined thereby. The antibodies of the invention can exhibit a specific binding affinity for hTRT of at least about  $10^7$ ,  $10^8$ ,  $10^9$ , or  $10^{10}$  M<sup>-1</sup>, and may be polyclonal, monoclonal, recombinant or otherwise produced. The invention also provides anti-hTRT antibodies that recognize an hTRT conformational epitope (e.g., an epitope on the surface of the hTRT protein or  
20 a telomerase RNP). Likely conformational epitopes can be identified, if desired, by computer-assisted analysis of the hTRT protein sequence, comparison to the conformation of related reverse transcriptases such as the p66 subunit of HIV-1 (see, e.g., **Figure 3**), or empirically. Anti-hTRT antibodies that recognize conformational epitopes have utility, *inter alia*, in detection and purification of human telomerase and  
25 in the diagnosis and treatment of human disease.

For the production of anti-hTRT antibodies, hosts such as goats, sheep, cows, guinea pigs, rabbits, rats, or mice, may be immunized by injection with hTRT protein or any portion, fragment or oligopeptide thereof which retains immunogenic properties. In selecting hTRT polypeptides for antibody induction, one need not retain  
30 biological activity; however, the protein fragment, or oligopeptide must be



immunogenic, and preferably antigenic. Immunogenicity can be determined by injecting a polypeptide and adjuvant into an animal (e.g., a rabbit) and assaying for the appearance of antibodies directed against the injected polypeptide (see, e.g., Harlow and Lane, ANTIBODIES: A LABORATORY MANUAL, COLD SPRING HARBOR

5 LABORATORY, New York (1988) which is incorporated in its entirety and for all purposes, e.g., at Chapter 5). Peptides used to induce specific antibodies typically have an amino acid sequence consisting of at least five amino acids, preferably at least 8 amino acids, more preferably at least 10 amino acids. Usually they will mimic or have substantial sequence identity to all or a contiguous portion of the amino acid sequence  
10 of the protein of **SEQ. ID. NO: 2**. Short stretches of hTRT protein amino acids may be fused with those of another protein, such as keyhole limpet hemocyanin, and an anti-hTRT antibody produced against the chimeric molecule. Depending on the host species, various adjuvants may be used to increase immunological response.

The antigen is presented to the immune system in a fashion determined  
15 by methods appropriate for the animal. These and other parameters are generally well known to immunologists. Typically, injections are given in the footpads, intramuscularly, intradermally, perilymph nodally or intraperitoneally. The immunoglobulins produced by the host can be precipitated, isolated and purified by routine methods, including affinity purification.

20 Illustrative examples of immunogenic hTRT peptides include are provided in **Example 8**. In addition, **Example 8** describes the production and use of anti-hTRT polyclonal antibodies.

#### A) MONOCLONAL ANTIBODIES

25 Monoclonal antibodies to hTRT proteins and peptides may be prepared in accordance with the methods of the invention using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (*Nature* 256:495 [1975]), the human B-cell hybridoma technique (Kosbor  
30 et al., 1983, *Immunol. Today* 4:72; Cote et al., 1983, *Proc. Natl. Acad. Sci. USA*,

80:2026), and the EBV-hybridoma technique (Cole et al., MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R Liss Inc, New York NY, pp 77-96 [1985]).

In one embodiment, appropriate animals are selected and the appropriate immunization protocol followed. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine is well known and can be accomplished by, for example, immunizing an animal with a preparation containing hTRT or fragments thereof. In one method, after the appropriate period of time, the spleens of the animals are excised and individual spleen cells are fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone (e.g., hybridoma) are tested for the production of an appropriate antibody specific for the desired region of the antigen. Techniques for producing antibodies are well known in the art. See, e.g., Goding et al., MONOCLONAL ANTIBODIES: PRINCIPLES AND PRACTICE (2D ED.) Acad. Press, N.Y., and Harlow and Lane, *supra*, each of which is incorporated in its entirety and for all purposes. Other suitable techniques involve the *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively, to selection of libraries of antibodies in phage or similar vectors (see, *infra*).

## **B) HUMAN ANTIBODIES**

In another aspect of the invention, human antibodies against an hTRT polypeptide are provided. Human monoclonal antibodies against a known antigen can also be made using transgenic animals having elements of a human immune system (see, e.g., U.S. Patent Nos. 5,569,825 and 5,545,806, both of which are incorporated by reference in their entirety for all purposes) or using human peripheral blood cells (Casali et al., 1986, *Science* 234:476). Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody.

In an alternative embodiment, human antibodies to a hTRT polypeptide can be produced by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., 1989, *Science* 246:1275, which is incorporated

by reference. Antibodies binding to the hTERT polypeptide are selected. Sequences encoding such antibodies (or a binding fragments) are then cloned and amplified. The protocol described by Huse is often used with phage-display technology.

5

### **C) HUMANIZED OR CHIMERIC ANTIBODIES**

The invention also provides anti-hTERT antibodies that are made chimeric, human-like or humanized, to reduce their potential antigenicity, without reducing their affinity for their target. Preparation of chimeric, human-like and humanized antibodies have been described in the art (see, e.g., U.S. Patent Nos. 10 5,585,089 and 5,530,101; Queen, et al., 1989, *Proc. Nat'l Acad. Sci. USA* 86:10029; and Verhoeyan et al., 1988, *Science* 239:1534; each of which are incorporated by reference in their entirety and for all purposes). Humanized immunoglobulins have variable framework regions substantially from a human immunoglobulin (termed an acceptor immunoglobulin) and complementarity determining regions substantially from 15 a non-human (e.g., mouse) immunoglobulin (referred to as the donor immunoglobulin). The constant region(s), if present, are also substantially from a human immunoglobulin.

In some applications, such as administration to human patients, the humanized (as well as human) anti-hTERT antibodies of the present invention offer several advantages over antibodies from murine or other species: (1) the human 20 immune system should not recognize the framework or constant region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody; (2) because the effector portion of the humanized antibody is human, it may interact better with other parts of the human immune system; 25 and (3) injected humanized antibodies have a half-life essentially equivalent to naturally occurring human antibodies, allowing smaller and less frequent doses than antibodies of other species.

30

### **D) PHAGE DISPLAY**

The present invention also provides anti-hTERT antibodies (or binding

compositions) produced by phage display methods (see, e.g., Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047; and Vaughan *et al.*, 1996, *Nature Biotechnology*, 14: 309; each of which is incorporated by reference in its entirety for all purposes). In these methods, libraries of phage are produced in which members display different antibodies on their outer surfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to an hTERT polypeptide.

In a variation of the phage-display method, humanized antibodies having the binding specificity of a selected murine antibody can be produced. In this method, either the heavy or light chain variable region of the selected murine antibody is used as a starting material. If, for example, a light chain variable region is selected as the starting material, a phage library is constructed in which members displays the same light chain variable region (i.e., the murine starting material) and a different heavy chain variable region. The heavy chain variable regions are obtained from a library of rearranged human heavy chain variable regions. A phage showing strong specific binding for the hTERT polypeptide (e.g., at least  $10^8$  and preferably at least  $10^9$  M<sup>-1</sup>) is selected. The human heavy chain variable region from this phage then serves as a starting material for constructing a further phage library. In this library, each phage displays the same heavy chain variable region (i.e., the region identified from the first display library) and a different light chain variable region. The light chain variable regions are obtained from a library of rearranged human variable light chain regions. Again, phage showing strong specific binding are selected. These phage display the variable regions of completely human anti-hTERT antibodies. These antibodies usually have the same or similar epitope specificity as the murine starting material.

#### E) HYBRID ANTIBODIES

The invention also provides hybrid antibodies that share the specificity of antibodies against an hTERT polypeptide but are also capable of specific binding to a second moiety. In such hybrid antibodies, one heavy and light chain pair is usually from an anti-hTERT antibody and the other pair from an antibody raised against another epitope or protein. This results in the property of multi-functional valency, i.e., ability to bind at least two different epitopes simultaneously, where at least one epitope is the epitope to which the anti-complex antibody binds. Such hybrids can be formed by fusion of hybridomas producing the respective component antibodies, or by recombinant techniques.

Immunoglobulins of the present invention can also be fused to functional regions from other genes (e.g., enzymes) to produce fusion proteins (e.g., immunotoxins) having useful properties.

#### **F) ANTI-IDIOTYPIC ANTIBODIES**

Also useful are anti-idiotypic antibodies which can be isolated by the above procedures. Anti-idiotypic antibodies may be prepared by, for example, immunization of an animal with the primary antibody (i.e., anti-hTERT antibodies or hTERT-binding fragments thereof). For anti-hTERT antibodies, anti-idiotypic antibodies whose binding to the primary antibody is inhibited by a hTERT polypeptide or fragments thereof are selected. Because both the anti-idiotypic antibody and the hTERT polypeptide or fragments thereof bind the primary immunoglobulin, the anti-idiotypic immunoglobulin may represent the "internal image" of an epitope and thus may substitute for the hTERT polypeptide in assays or may be used to bind (i.e., inactivate) anti-hTERT antibodies, e.g., in a patient. Anti-idiotypic antibodies may also interact with telomerase associated proteins. Administration of such antibodies could affect telomerase function by titrating out hTERT-associated proteins.

#### **G) GENERAL**

The antibodies of the invention may be of any isotype, e.g., IgM, IgD, IgG, IgA, and IgE, with IgG, IgA and IgM often preferred. Humanized antibodies may

comprise sequences from more than one class or isotype.

In another embodiment of the invention, fragments of the intact antibodies described above are provided. Typically, these fragments can compete with the intact antibody from which they were derived for specific binding to the hTRT polypeptide, and bind with an affinity of at least  $10^7$ ,  $10^8$ ,  $10^9$   $M^{-1}$ , or  $10^{10}$   $M^{-1}$ . Antibody fragments include separate heavy chains, light chains, Fab, Fab' F(ab')<sub>2</sub>, Fabc, and Fv. Fragments can be produced by enzymatic or chemical separation of intact immunoglobulins. For example, a F(ab')<sub>2</sub> fragment can be obtained from an IgG molecule by proteolytic digestion with pepsin at pH 3.0-3.5 using standard methods such as those described in Harlow and Lane, *supra*. Fab fragments may be obtained from F(ab')<sub>2</sub> fragments by limited reduction, or from whole antibody by digestion with papain in the presence of reducing agents (*see generally*, Paul, W., ed FUNDAMENTAL IMMUNOLOGY 2ND Raven Press, N.Y., 1989, Ch. 7, incorporated by reference in its entirety for all purposes). Fragments can also be produced by recombinant DNA techniques. Segments of nucleic acids encoding selected fragments are produced by digestion of full-length coding sequences with restriction enzymes, or by *de novo* synthesis. Often fragments are expressed in the form of phage-coat fusion proteins.

Many of the immunoglobulins described above can undergo non-critical amino-acid substitutions, additions or deletions in both the variable and constant regions without loss of binding specificity or effector functions, or intolerable reduction of binding affinity (i.e., below about  $10^7$   $M^{-1}$ ). Usually, immunoglobulins incorporating such alterations exhibit substantial sequence identity to a reference immunoglobulin from which they were derived. A mutated immunoglobulin can be selected having the same specificity and increased affinity compared with a reference immunoglobulin from which it was derived. Phage-display technology offers useful techniques for selecting such immunoglobulins. *See*, e.g., Dower et al., WO 91/17271 McCafferty et al., WO 92/01047; and Huse, WO 92/06204.

The antibodies of the present invention can be used with or without modification. Frequently, the antibodies will be labeled by joining, either covalently or non-covalently, a detectable label. As labeled binding entities, the antibodies of the

invention are particularly useful in diagnostic applications.

The anti-hTERT antibodies of the invention can be purified using well known methods. The whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified using the methods and reagents of the present invention in accordance with standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (*see generally* Scopes, PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE 3RD EDITION (Springer-Verlag, N.Y., 19\_\_). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity is often most preferred.

## VI. PURIFICATION OF HUMAN TELOMERASE

The present invention provides isolated human telomerase of unprecedented purity. In particular, the present invention provides: purified hTERT of recombinant or nonrecombinant origin; purified hTERT-hTR complexes (i.e., RNPs) of recombinant, nonrecombinant, or mixed origin, optionally comprising one or more telomerase-associated proteins; purified naturally occurring human telomerase; and the like. Moreover, the invention provides methods and reagents for partially, substantially or highly purifying the above-molecules and complexes, including variants, fusion proteins, naturally occurring proteins, and the like (collectively referred to as "hTERT and/or hTERT complexes").

Prior to the present disclosure, attempts had been made to purify the telomerase enzyme complex to homogeneity had met with limited success (see, e.g., copending U.S. patent application Serial Nos. 08/833,377, filed April 4, 1997 and 08/510,736, filed August 4, 1995, and PCT application No. 97/06012, filed April 4, 1997, all of which are incorporated herein by reference, for useful purification methods). The methods provided in the aforelisted applications provide purification of telomerase by approximately up to 60,000-fold or more compared to crude cell extracts. The present invention provides hTERT and hTERT complexes of even greater purity, in part, by virtue of: the novel immunoaffinity reagents (e.g., anti-hTERT antibodies) of

the present invention, and/or the reagents, cells, and methods provided herein for recombinant expression hTERT. Recombinant expression of hTERT and hTERT complexes facilitates purification because the desired molecules can be produced at much higher levels than found in most expressing cells occurring in nature, and/or because the recombinant hTERT molecules can be modified (e.g., by fusion with an epitope tag) such that it may be easily purified.

It will be recognized that naturally occurring telomerase may be purified from any telomerase-positive cell, and recombinant hTERT and hTERT complexes may be expressed and purified, *inter alia*, using any of the *in vitro*, *in vivo*, *ex vivo*, or plant or animal expression systems disclosed *supra*, or others/systems known in the art.

In one embodiment, the hTERT, telomerase and other compositions of the invention are purified using an immunoaffinity step, alone or in combination with other purification steps. Typically, an immobilized or immobilizable anti-hTERT antibody, as provided by the present invention, is contacted with a sample, such as a cell lysate, that contains the desired hTERT or hTERT-containing complex under conditions in which anti-hTERT antibody binds the hTERT antigen. After removal of the unbound components of the sample by methods well known in the art, the hTERT composition may be eluted, if desired, from the antibody, in substantially pure form. In one embodiment, immunoaffinity chromatography methods well known in the art are used (see, e.g., Harlow and Lane, *supra*; and Ausubel, *supra*; Hermanson et al., 1992, IMMOBILIZED AFFINITY LIGAND TECHNIQUES (Academic Press, San Diego)) in accordance with the methods of the invention. In another illustrative embodiment, immunoprecipitation of anti-hTERT-immunoglobulin-hTERT complexes is carried out using immobilized Protein A. Numerous variations and alternative immunoaffinity purification protocols suitable for use in accordance with the methods and reagents of the invention are well-known to those of skill.

In another embodiment, recombinant hTERT proteins can, as a consequence of their high level of expression, be purified using routine protein purification methods, such as ammonium sulfate precipitation, affinity columns (e.g., immunoaffinity), size-exclusion, anion and cation exchange chromatography, gel



electrophoresis and the like (see, generally, R. Scopes, PROTEIN PURIFICATION, Springer-Verlag, N.Y. (1982) and Deutscher, METHODS IN ENZYMOLOGY VOL. 182: GUIDE TO PROTEIN PURIFICATION, Academic Press, Inc. N.Y. (1990)) instead of, or in addition to, immunoaffinity methods. Cation exchange methods can be particularly useful due to the basic pI of the hTERT protein. For example, immobilized phosphate may be used as a cation exchange functional group (e.g., P-11 Phosphocellulose, Whatman catalog #4071 or Cellulose Phosphate, Sigma catalog #C 3145).

Immobilized phosphate has two advantageous features for hTERT purification - it is a cation exchange resin, and it shows physical resemblance to the phosphate backbone of nucleic acid. This may allow pseudo-affinity chromatography since hTERT binds hTERT and telomeric DNA. Other non-specific nucleic acid affinity chromatography methods are also useful for purification (e.g., copending U.S. patent application Serial No. 08/833,377; Alberts et al., 1971, *Methods Enzymol.* 21:198; Arnt-Jovin et al., 1975, *Eur. J. Biochem.* 54:411; Pharmacia catalog #27-5575-02). Further exploitation of this likely binding function of hTERT would include the use of specific nucleic acid (e.g., primer or hTERT) affinity chromatography for purification (Chodosh et al., 1986, *Mol. Cell. Biol.* 6:4723; Wu et al., 1987, *Science* 238:1247; Kadonaga, 1991, *Methods Enzymol.* 208:10); immobilized Cibricon Blue Dye, which shows physical resemblance to nucleotides, is another useful resin for hTERT purification (Pharmacia catalog #17-0948-01 or Sigma catalog #C 1285), due to hTERT binding of nucleotides (e.g., as substrates for DNA synthesis).

In one embodiment, hTERT proteins are isolated directly from an *in vitro* or *in vivo* expression system in which other telomerase components are not coexpressed. It will be recognized that isolated hTERT protein may also be readily obtained from purified human telomerase or hTERT complexes, for example, by disrupting the telomerase RNP (e.g., by exposure to a mild or other denaturant) and separating the RNP components (e.g., by routine means such as chromatography or immunoaffinity chromatography).

Telomerase purification may be monitored using a telomerase activity assay (e.g., the TRAP assay, conventional assay, or primer-binding assay), by

measuring the enrichment of hTERT (e.g., by ELISA), by measuring the enrichment of hTR, or other methods known in the art.

The purified human telomerase, hTERT proteins, and hTERT complexes provided by the present invention are, in one embodiment, highly purified (i.e., at least about 90% homogeneous, more often at least about 95% homogeneous). Homogeneity can be determined by standard means such as SDS-polyacrylamide gel electrophoresis and other means known in the art (see, e.g., Ausubel et al, *supra*). It will be understood that, although highly purified human telomerase, hTERT protein, or hTERT complexes are sometimes desired, substantially purified (e.g., at least about 75% homogeneous) or partially purified (e.g., at least about 20% homogeneous) human telomerase, hTERT protein, or hTERT complexes are useful in many applications, and are also provided by the present invention. For example, partially purified telomerase is useful for screening test compounds for telomerase modulatory activity, and other uses (see, e.g., copending U.S. patent application serial no. \_\_\_\_\_ (Attorney docket no. 015389-002500 filed August 14, 1997, cited *supra* and U.S. Patent No. 5,645,986, USSN 08/151,477, filed 12 November 1993, and USSN 08/288,501, filed 10 August 1994).

## VII. TREATMENT OF TELOMERASE-RELATED DISEASE

### A) INTRODUCTION

The present invention provides hTERT polynucleotides, polypeptides, and antibodies useful for the treatment of human diseases and disease conditions. The recombinant and synthetic hTERT gene products (protein and mRNA) of the invention can be used to create or elevate telomerase activity in a cell, as well as to inhibit telomerase activity in cells in which it is not desired. Thus, inhibiting, activating or otherwise altering a telomerase activity (e.g., telomerase catalytic activity, fidelity, processivity, telomere binding, *etc.*) in a cell can be used to change the proliferative capacity of the cell. For example, reduction of telomerase activity in an immortal cell, such as a malignant tumor cell, can render the cell mortal. Conversely, increasing the telomerase activity in a mortal cell (e.g., most human somatic cells) can increase the proliferative capacity of the cell. For example, expression of hTERT protein in dermal

fibroblasts, thereby increasing telomere length, will result in increased fibroblast proliferative capacity; such expression can slow or reverse the age-dependent slowing of wound closure (see, e.g., West, 1994, *Arch. Derm.* 130:87).

Thus, in one aspect, the present invention provides reagents and methods useful for treating diseases and conditions characterized by the presence, absence, or amount of human telomerase activity in a cell and that are susceptible to treatment using the compositions and methods disclosed herein. These diseases include, as described more fully below, cancers, other diseases of cell proliferation (particularly diseases of aging), immunological disorders, infertility (or fertility), and others.

## B) TREATMENT OF CANCER

The present invention provides methods and compositions for reducing telomerase activity in tumor cells and for treating cancer. Cancer cells (e.g., malignant tumor cells) that express telomerase activity (telomerase-positive cells) can be mortalized by decreasing or inhibiting the endogenous telomerase activity. Moreover, because telomerase levels correlate with disease characteristics such as metastatic potential (e.g., U.S. Patent No. 5,639,613; 5,648,215; 5,489,508; Pandita et al., 1996, *Proc. Am. Ass. Cancer Res.* 37:559), any reduction in telomerase activity could reduce the aggressive nature of a cancer to a more manageable disease state (increasing the efficacy of traditional interventions).

The invention provides compositions and methods useful for treatment of cancers of any of a wide variety of types, including solid tumors and leukemias. Types of cancer that may be treated include (but are not limited to): adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid; melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhus, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders; leukemia (e.g., B-cell, mixed-cell, null-cell, T-cell, T-cell chronic, HTLV-II-associated,

lyphocytic acute, lymphocytic chronic, mast-cell, and myeloid); histiocytosis malignant; Hodgkin's disease; immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma;

5 liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; Ewing's sarcoma; synovioma; adenofibroma; adenolymphoma; carcinosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor; adenocarcinoma; adenoma; cholangioma; cholesteatoma;

10 cylindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor; gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; leydig cell tumor; papilloma; sertoli cell tumor; theca cell tumor; leiomyoma; leiomyosarcoma; myoblastoma; myoma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma;

15 neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma; paraganglioma; paraganglioma nonchromaffin; angiokeratoma; angiolymphoid hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma; lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma;

20 chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma (e.g., Ewing's, experimental, Kaposi's, and mast-cell); neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous

25 system, acoustic, pelvic, respiratory tract, and urogenital); neurofibromatosis, and cervical dysplasia). The invention provides compositions and methods useful for treatment of other conditions in which cells have become immortalized or hyperproliferative, e.g., by disregulation (e.g., abnormally high expression) of hTERT, telomerase enzyme, or activity.

30 The present invention further provides compositions and methods for

prevention of cancers, including anti-hTERT vaccines, gene therapy vectors that prevent telomerase activation, and gene therapy vectors that result in specific death of telomerase-positive cells. In a related aspect, the gene replacement therapy methods described below may be used for "treating" a genetic predilection for cancers.

5

### C) TREATMENT OF OTHER CONDITIONS

The present invention also provides compositions and methods useful for treatment of diseases and disease conditions (in addition to cancers) characterized by under- or over-expression of telomerase or hTERT gene products. Examples include:

10 diseases of cell proliferation, diseases resulting from cell senescence (particularly diseases of aging), immunological disorders, infertility, diseases of immune dysfunction, and others.

Certain diseases of aging are characterized by cell senescence-associated changes due to reduced telomere length (compared to younger cells), resulting from the

15 absence (or much lower levels) of telomerase activity in the cell. Decreased telomere length and decreased replicative capacity contribute to diseases such as those described below. Telomerase activity and telomere length can be increased by, for example, increasing levels of hTERT gene products (protein and mRNA) in the cell. A partial

20 listing of conditions associated with cellular senescence in which hTERT may be under-expressed (compared to younger cells) includes Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke; age-related diseases of the integument such as dermal atrophy, elastolysis and skin wrinkling, sebaceous gland hyperplasia, senile lentigo, graying of hair and hair loss, chronic skin ulcers, and age-related impairment of wound healing; degenerative joint disease; osteoporosis; age-related immune system

25 impairment (e.g., involving cells such as B and T lymphocytes, monocytes, neutrophils, eosinophils, basophils, NK cells and their respective progenitors); age-related diseases of the vascular system including atherosclerosis, calcification, thrombosis, and aneurysms; diabetes, muscle atrophy, respiratory diseases, diseases of the liver and GI tract, metabolic diseases, endocrine diseases (e.g., disorders of the

30 pituitary and adrenal gland), reproductive diseases, and age-related macular

degeneration. These diseases and conditions can be treated by increasing the levels of hTERT gene products in the cell to increase telomere length, thereby restoring or imparting greater replicative capacity to the cell. Such methods can be carried out on cells cultured *ex vivo* or cells *in vivo*. In one embodiment, the cells are first treated to lengthen telomerase and then treated to inactivate the hTERT gene and telomerase activity.

In a preferred embodiment, telomerase activity is generated by a vector of the invention in an embryonic germ or stem cell (see USSN 08/591,246, filed 18 January 1996; USSN 08/376,327, filed 20 January 1995; Pederson et al.; USSN \_\_\_\_\_, filed 13 June 1997, a CIP of USSN 08/665,217, filed 14 June 1996; and USSN 08/829,372, filed 31 March 1997) prior to or during differentiation.

The present invention also provides methods and composition useful for treating infertility. Human germline cells (e.g., spermatogonia cells, their progenitors or descendants) are capable of indefinite proliferation and characterized by high telomerase activity. Abnormal or diminished levels of hTERT gene products can result, for example, in inadequate or abnormal production of spermatozoa, leading to infertility or disorders of reproduction. Accordingly, "telomerase-based" infertility can be treated using the methods and compositions described herein to increase telomerase levels. Similarly, because inhibition of telomerase may negatively impact spermatogenesis, oogenesis, and sperm and egg viability, the telomerase inhibitory compositions of the invention can have contraceptive effects when used to reduce hTERT gene product levels in germline cells.

Further, the invention provides methods and composition useful for decreasing the proliferative potential of telomerase-positive cells such as activated lymphocytes and hematopoietic stem cells by reducing telomerase activity. Thus, the invention provide means for effecting immunosuppression. Conversely, the methods and reagents of the invention are useful for increasing telomerase activity and proliferative potential in cells, such as stem cells, that express a low level of telomerase or no telomerase prior to therapeutic intervention.

**D) MODES OF INTERVENTION**

As is clear from the foregoing discussion, modulation of the level of telomerase or telomerase activity of a cell can have a profound effect on the proliferative potential of the cell, and so has great utility in treatment of disease. As is also clear, this modulation may be either a decrease in telomerase activity or an increase in activity. The telomerase modulatory molecules of the invention can act through a number of mechanisms; some of these are described in this and the following subsections to aid the practitioner in selecting therapeutic agents. However, applicants do not intend to be limited to any particular mechanism of action for the novel therapeutic compounds, compositions and methods described herein.

Telomerase activity may be decreased through any of several mechanisms or combinations of mechanisms. One mechanism is the reduction of hTERT gene expression to reduce telomerase activity. This reduction can be at the level of transcription of the hTERT gene into mRNA, processing (e.g., splicing), nuclear transport or stability of mRNA, translation of mRNA to produce hTERT protein, or stability and function of hTERT protein. Another mechanism is interference with one or more activities of telomerase (e.g., the reverse transcriptase catalytic activity, or the hTR-binding activity) using inhibitory nucleic acids, polypeptides, or other agents (e.g., mimetics, small molecules, drugs and pro-drugs) that can be identified using the methods or are provided by compositions disclosed herein. Other mechanisms include sequestration of hTR and/or telomerase associated proteins, and interference with the assembly of the telomerase RNP from its component subunits. In a related mechanism, an hTERT promoter sequence is operably linked to a gene encoding a toxin and introduced into a cell; if or when hTERT transcriptional activators are expressed or activated in the cell, the toxin will be expressed, resulting in specific cell killing.

A related method for reducing the proliferative capacity of a cell involves introducing a hTERT variant with low fidelity (i.e., one with a high, e.g., greater than 1%, error rate) such that aberrant telomeric repeats. These aberrant repeats affect telomere protein binding and lead to chromosomal rearrangements and aberrations and/or lead to cell death.

Similarly, telomerase activity may be increased through any of several mechanisms, or a combination of mechanisms. These include increasing the amount of hTERT in a cell. Usually this is carried out by introducing an hTERT polypeptide-encoding polynucleotide into the cell (e.g., a recombinantly produced polypeptide comprising an hTERT DNA sequence operably linked to a promoter, or a stable hTERT mRNA). Alternatively, a catalytically active hTERT polypeptide can itself be introduced into a cell or tissue, e.g., by microinjection or other means known in the art. In other mechanisms, expression from the endogenous hTERT gene or the stability of hTERT gene products in the cell can be increased. Telomerase activity in a cell can also be increased by interfering with the interaction of endogenous telomerase inhibitors and the telomerase RNP, or endogenous hTERT transcription repressors and the hTERT gene, and other means apparent to those of skill upon review of this disclosure.

## **E) INTERVENTION AGENTS**

### **1) TRT PROTEINS & PEPTIDES**

In one embodiment, the invention provides telomerase modulatory polypeptides (i.e., proteins, polypeptides, and peptides) that increase or reduce telomerase activity which can be introduced into a target cell directly (e.g., by injection, liposome-mediated fusion, application of a hydrogel to the tumor [e.g., melanoma] surface, fusion or attachment to herpes virus structural protein VP22, and other means described herein and known in the art). In a second embodiment, telomerase modulatory proteins and peptides of the invention are expressed in a cell by introducing a nucleic acid (e.g., a DNA expression vector or mRNA) encoding the desired protein or peptide into the cell. Expression may be either constitutive or inducible depending on the vector and choice of promoter (*see* discussion below). Messenger RNA preparations encoding hTERT are especially useful when only transient expression (e.g., transient activation of telomerase) is desired. Methods for introduction and expression of nucleic acids into a cell are well known in the art (also, see elsewhere in this specification, e.g., sections on oligonucleotides, gene therapy methods).

In one aspect of the invention, a telomerase modulatory polypeptide that



increases telomerase activity in a cell is provided. In one embodiment, the polypeptide is a catalytically active hTRT polypeptide capable of directing the synthesis (in conjunction with an RNA template such as hTR) of human telomeric DNA. This activity can be measured, as discussed above, e.g., using a telomerase activity assay such as a TRAP assay. In one embodiment, the polypeptide is a full-length hTRT protein, having a sequence of, or substantially identical to, the sequence of 1132 residues of **SEQ. ID. No: 2**. In another embodiment, the polypeptide is a variant of the hTRT protein of **SEQ. ID. No: 2**, such as a fusion polypeptide, derivatized polypeptide, truncated polypeptide, conservatively substituted polypeptide, or the like. A fusion or derivatized protein may include a targeting moiety that increases the ability of the polypeptide to traverse a cell membrane or causes the polypeptide to be preferentially delivered to a specified cell type (e.g., liver cells or tumor cells) or cell compartment (e.g., nuclear compartment). Examples of targeting moieties include lipid tails, amino acid sequences such as antennopodia peptide (see USSN 08/838,545, filed 9 April 1997) or a nuclear localization signal (NLS; e.g., *Xenopus* nucleoplasmin Robbins et al., 1991, *Cell* 64:615). Naturally occurring hTRT protein (e.g., having a sequence of, or substantially identical to, **SEQ. ID. NO: 2**) acts in the cell nucleus. Thus, it is likely that one or more subsequences of **SEQ. ID. NO: 2**, such as residues 193-196 (PRRR) and residues 235-240 (PKRPRR) act as a nuclear localization signal. The small regions are likely NLSs based on the observation that many NLSs comprise a 4 residue pattern composed of basic amino acids (K or R), or composed of three basic amino acids (K or R) and H or P; a pattern starting with P and followed within 3 residues by a basic segment containing 3 K or R residues out of 4 residues. See Nakai et al., 1992, *Genomics* 14:897). Deletion of one or both of these sequences and/or additional localization sequences is expected to interfere with hTRT transport to the nucleus and/or increase hTRT turnover, and is useful for preventing access of telomerase to its nuclear substrates and decreasing proliferative potential. Moreover, a variant hTRT polypeptide lacking NLS may assemble into an RNP that will not be able to maintain telomere length, because the resulting enzyme cannot enter the nucleus.

The hTRT polypeptides of the invention will typically be associated in

the target cell with a telomerase RNA, such as hTR, when they are used to increase telomerase activity in a cell. In one embodiment, an introduced hTRT polypeptide associates with an endogenous hTR to form a catalytically active RNP (e.g., an RNP comprising the hTR and a full-length polypeptide having a sequence of SEQ. ID. NO.

5 2). The RNP so-formed may also associate with other, e.g., telomerase-associated, proteins. In other embodiments, telomerase RNP (containing hTRT protein, hTR and optionally other components) is introduced as a complex to the target cell.

In a related embodiment, an hTRT expression vector is introduced into a cell (or progeny of a cell) into which a telomerase RNA (e.g., hTR) expression vector is  
10 simultaneously, subsequently or previously introduced. In this embodiment, hTRT protein and telomerase RNA are coexpressed in the cell and assemble to form a telomerase RNP. A preferred telomerase RNA is hTR. An expression vector useful for expression of hTR in a cell is described *supra* (see U.S. Patent 5,583,016). In yet another embodiment, the hTRT polypeptide and hTR RNA (or equivalent) are  
15 associated *in vitro* to form a complex, which is then introduced into the target cells.

In another aspect, the invention provides hTRT polypeptides useful for reducing telomerase activity in a cell. As above, these “inhibitory” polypeptides can be introduced directly, or by expression of recombinant nucleic acids in the cell. It will be recognized that peptide mimetics or polypeptides comprising nonstandard amino acids  
20 (i.e., other than the 20 amino acids encoded by the genetic code or their normal derivatives) will usually be introduced directly.

In one embodiment, inhibition of telomerase activity results from the sequestration of a component required for accurate telomere elongation. Examples of such components are hTRT and hTR. Thus, administration of a polypeptide that binds  
25 hTR, but which does not have telomerase catalytic activity, can reduce endogenous telomerase activity in the cell. In a related embodiment, the hTRT polypeptide may bind a cell component other than hTR, such as one or more telomerase-associated proteins, thereby interfering with telomerase activity in the cell.

In another embodiment, hTRT polypeptides of the invention interfere  
30 (e.g., by competition) with the interaction of endogenously expressed hTRT protein and

another cellular component required for telomerase function, such as hTR, telomeric DNA, telomerase-associated proteins, telomere-associated proteins, telomeres, cell cycle control proteins, DNA repair enzymes, histone or non-histone chromosomal proteins, or others.

5                   In selecting molecules (e.g., polypeptides) of the invention that affect the interaction of endogenously expressed hTERT protein and other cellular components, one may prefer molecules that include one or more of the conserved motifs of the hTERT protein, as described herein. The evolutionary conservation of these regions indicates the important function in the proper functioning of human telomerase contributed by  
10                   these motifs, and the motifs are thus generally useful sites for changing hTERT protein function to create variant hTERT proteins of the invention. Thus, variant hTERT polypeptides having mutations in conserved motifs will be particular useful.

                  In another embodiment, expression of the endogenous hTERT gene is repressed by introduction into the cell of a large amount of hTERT polypeptide (e.g.,  
15                   typically at least about 2-fold more than the endogenous level, more often at least about 10- to about 100-fold) which acts via a feedback loop to inhibit transcription of the hTERT gene processing of the hTERT pre-mRNA, translation of the hTERT mRNA, or assembly and transport of the telomerase RNP.

## 20                   2) OLIGONUCLEOTIDES

### a) ANTISENSE CONSTRUCTS

                  The invention provides methods and antisense oligonucleotide or polynucleotide reagents which can be used to reduce expression of hTERT gene products *in vitro* or *in vivo*. Administration of the antisense reagents of the invention to a target  
25                   cell results in reduced telomerase activity, and is particularly useful for treatment of diseases characterized by high telomerase activity (e.g., cancers). Without intending to be limited to any particular mechanism, it is believed that antisense oligonucleotides bind to, and interfere with the translation of, the sense hTERT mRNA. Alternatively, the antisense molecule may render the hTERT mRNA susceptible to nuclease digestion,  
30                   interfere with transcription, interfere with processing, localization or otherwise with

RNA precursors ("pre-mRNA"), repress transcription of mRNA from the hTRT gene, or act through some other mechanism. However, the particular mechanism by which the antisense molecule reduces hTRT expression is not critical.

5 The antisense polynucleotides of the invention comprise an antisense sequence of at least 7 to 10 or more nucleotides that specifically hybridize to a sequence from mRNA encoding human TRT or mRNA transcribed from the hTRT gene. More often, the antisense polynucleotide of the invention is from about 10 to about 50 nucleotides in length or from about 14 to about 35 nucleotides in length. In other embodiments, antisense polynucleotides are polynucleotides of less than about 100 nucleotides or less than about 200 nucleotides. In general, the antisense polynucleotide should be long enough to form a stable duplex but short enough, depending on the mode of delivery, to administer *in vivo*, if desired. The minimum length of a polynucleotide required for specific hybridization to a target sequence depends on several factors, such as G/C content, positioning of mismatched bases (if 15 any), degree of uniqueness of the sequence as compared to the population of target polynucleotides, and chemical nature of the polynucleotide (e.g., methylphosphonate backbone, peptide nucleic acid, phosphorothioate), among others.

Generally, to assure specific hybridization, the antisense sequence is substantially complementary to the target hTRT mRNA sequence. In certain 20 embodiments, the antisense sequence is exactly complementary to the target sequence. The antisense polynucleotides may also include, however, nucleotide substitutions, additions, deletions, transitions, transpositions, or modifications so long as specific binding to the relevant target sequence corresponding to hTRT RNA or its gene is retained as a functional property of the polynucleotide.

25 In one embodiment, the antisense sequence is complementary to relatively accessible sequences of the hTRT mRNA (e.g., relatively devoid of secondary structure). This can be determined by analyzing predicted RNA secondary structures using, for example, the MFOLD program (Genetics Computer Group, Madison WI) and testing *in vitro* or *in vivo* as is known in the art. Examples of 30 oligonucleotides that may be tested in cells for antisense suppression of hTRT function

are those capable of hybridizing to (i.e., substantially complementary to) the following positions from **SEQ. ID. NO:1**: 40-60; 260-280; 500-520; 770-790; 885-905; 1000-1020 ; 1300-1320; 1520-1540; 2110-2130; 2295-2315; 2450-2470; 2670-2690; 3080-3110; 3140-3160; and 3690-3710. Another useful method for identifying effective antisense compositions uses combinatorial arrays of oligonucleotides (see, e.g., Milner et al., 1997, *Nature Biotechnology* 15:537).

The invention also provides an antisense polynucleotide that has sequences in addition to the antisense sequence (i.e., in addition to anti-hTERT-sense sequence). In this case, the antisense sequence is contained within a polynucleotide of longer sequence. In another embodiment, the sequence of the polypeptide consists essentially of, or is, the antisense sequence.

The antisense nucleic acids (DNA, RNA, modified, analogues, and the like) can be made using any suitable method for producing a nucleic acid, such as the methods disclosed herein. In one embodiment, for example, antisense RNA molecules of the invention may be prepared by *de novo* chemical synthesis or by cloning. For example, an antisense RNA that hybridizes to hTERT mRNA can be made by inserting (ligating) a hTERT DNA sequence (e.g., **Seq. ID No. 1**, or fragment thereof) in reverse orientation operably linked to a promoter in a vector (e.g., plasmid). Provided that the promoter and, preferably termination and polyadenylation signals, are properly positioned, the strand of the inserted sequence corresponding to the noncoding strand will be transcribed and act as an antisense oligonucleotide of the invention.

For general methods relating to antisense polynucleotides, see ANTISENSE RNA AND DNA, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). See also, Dagle et al., 1991, *Nucleic Acids Research*, 19:1805. For a review of antisense therapy, see, e.g., Uhlmann et al., *Chem. Reviews*, 90:543-584 (1990).

#### **b) TRIPLEX OLIGO- AND POLYNUCLEOTIDES**

The present invention provides oligo- and polynucleotides (e.g., DNA, RNA or PNA) that bind to double-stranded or duplex hTERT nucleic acids (e.g., in a

folded region of the hTRT RNA or in the hTRT gene), forming a triple helix-containing, or "triplex" nucleic acid. Triple helix formation results in inhibition of hTRT expression by, for example, preventing transcription of the hTRT gene, thus reducing or eliminating telomerase activity in a cell. Without intending to be bound by any particular mechanism, it is believed that triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules to occur.

Triplex oligo- and polynucleotides of the invention are constructed using the base-pairing rules of triple helix formation (see, e.g., Cheng et al., 1988, *J. Biol. Chem.* 263: 15110; Ferrin and Camerini-Otero, 1991, *Science* 354:1494; Ramdas et al., 1989, *J. Biol. Chem.* 264:17395; Strobel et al., 1991, *Science* 254:1639; and Rigas et al., 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83: 9591; each of which is incorporated herein by reference) and the hTRT mRNA and/or gene sequence. Typically, the triplex-forming oligonucleotides of the invention comprise a specific sequence of from about 10 to at least about 25 nucleotides or longer "complementary" to a specific sequence in the hTRT RNA or gene (i.e., large enough to form a stable triple helix, but small enough, depending on the mode of delivery, to administer *in vivo*, if desired). In this context, "complementary" means able to form a stable triple helix. In one embodiment, oligonucleotides are designed to bind specifically to the regulatory regions of the hTRT gene (e.g., the hTRT 5'-flanking sequence, promoters, and enhancers) or to the transcription initiation site, (e.g., between -10 and +10 from the transcription initiation site). For a review of recent therapeutic advances using triplex DNA, see Gee et al., in Huber and Carr, 1994, MOLECULAR AND IMMUNOLOGIC APPROACHES, Futura Publishing Co, Mt Kisco NY and Rininsland et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:5854, which are both incorporated herein by reference.

**c) RIBOZYMES**

The present invention also provides ribozymes useful for inhibition of telomerase activity. The ribozymes of the invention bind and specifically cleave and inactivate hTERT mRNA. Useful ribozymes can comprise 5'- and 3'-terminal sequences complementary to the hTERT mRNA and can be engineered by one of skill on the basis of the hTERT mRNA sequence disclosed herein (see PCT publication WO 93/23572, *supra*). Ribozymes of the invention include those having characteristics of group I intron ribozymes (Cech, 1995, *Biotechnology* 13:323 and others of hammerhead ribozymes (Edgington, 1992, *Biotechnology* 10:256).

Ribozymes of the invention include those having cleavage sites such as GUA, GUU and GUC. Other optimum cleavage sites for ribozyme-mediated inhibition of telomerase activity in accordance with the present invention include those described in PCT publications WO 94/02595 and WO 93/23569, both incorporated herein by reference. Short RNA oligonucleotides between 15 and 20 ribonucleotides in length corresponding to the region of the target hTERT gene containing the cleavage site can be evaluated for secondary structural features that may render the oligonucleotide more desirable. The suitability of cleavage sites may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays, or by testing for *in vitro* ribozyme activity in accordance with standard procedures known in the art.

As described by Hu et al., PCT publication WO 94/03596, incorporated herein by reference, antisense and ribozyme functions can be combined in a single oligonucleotide. Moreover, ribozymes can comprise one or more modified nucleotides or modified linkages between nucleotides, as described above in conjunction with the description of illustrative antisense oligonucleotides of the invention.

In one embodiment, the ribozymes of the invention are generated *in vitro* and introduced into a cell or patient. In another embodiment, gene therapy methods are used for expression of ribozymes in a target cell *ex vivo* or *in vivo*.

**d) ADMINISTRATION OF OLIGONUCLEOTIDES**

Typically, the therapeutic methods of the invention involve the administration of an oligonucleotide that functions to inhibit or stimulate telomerase activity under *in vivo* physiological conditions, and is relatively stable under those conditions for a period of time sufficient for a therapeutic effect. As noted above, modified nucleic acids may be useful in imparting such stability, as well as for targeting delivery of the oligonucleotide to the desired tissue, organ, or cell.

Oligo- and poly-nucleotides can be delivered directly as a drug in a suitable pharmaceutical formulation, or indirectly by means of introducing a nucleic acid into a cell, including liposomes, immunoliposomes, ballistics, direct uptake into cells, and the like as described herein. For treatment of disease, the oligonucleotides of the invention will be administered to a patient in a therapeutically effective amount. A therapeutically effective amount is an amount sufficient to ameliorate the symptoms of the disease or modulate telomerase activity in the target cell, e.g., as can be measured using a TRAP assay or other suitable assay of telomerase biological function. Methods useful for delivery of oligonucleotides for therapeutic purposes are described in U.S. Patent 5,272,065, incorporated herein by reference. Other details of administration of pharmaceutically active compounds are provided below. In another embodiment, oligo- and poly-nucleotides can be delivered using gene therapy and recombinant DNA expression plasmids of the invention.

### 3) GENE THERAPY

Gene therapy refers to the introduction of an otherwise exogenous polynucleotide which produces a medically useful phenotypic effect upon the (typically) mammalian cell(s) into which it is transferred. In one aspect, the present invention provides gene therapy methods and compositions for treatment of telomerase-associated conditions. In illustrative embodiments, gene therapy involves introducing into a cell a vector that expresses an hTERT gene product (such as an hTERT protein substantially similar to the hTERT polypeptide having a sequence of SEQ. ID. NO: 2, e.g., to increase telomerase activity, or an inhibitory hTERT polypeptide to reduce activity), expresses a nucleic acid having an hTERT gene or mRNA sequence (such as an



antisense RNA, e.g., to reduce telomerase activity), expresses a polypeptide or polynucleotide that otherwise affects expression of hTERT gene products (e.g., a ribozyme directed to hTERT mRNA to reduce telomerase activity), or replaces or disrupts an endogenous hTERT sequence (e.g., gene replacement and "gene knockout," respectively). Numerous other embodiments will be evident to one of skill upon review of the disclosure herein. In one embodiment, a vector encoding hTERT is also introduced. In another embodiment, vectors encoding telomerase-associated proteins are also introduced with or without a vector for hTERT.

Vectors useful in hTERT gene therapy can be viral or nonviral, and include those described *supra* in relation to the hTERT expression systems of the invention. It will be understood by those of skill in the art that gene therapy vectors may comprise promoters and other regulatory or processing sequences, such as are described in this disclosure. Usually the vector will comprise a promoter and, optionally, an enhancer (separate from any contained within the promoter sequences) that serve to drive transcription of an oligoribonucleotide, as well as other regulatory elements that provide for episomal maintenance or chromosomal integration and for high-level transcription, if desired. A plasmid useful for gene therapy can comprise other functional elements, such as selectable markers, identification regions, and other sequences. The additional sequences can have roles in conferring stability both outside and within a cell, targeting delivery of hTERT nucleotide sequences (sense or antisense) to a specified organ, tissue, or cell population, mediating entry into a cell, mediating entry into the nucleus of a cell and/or mediating integration within nuclear DNA. For example, aptamer-like DNA structures, or other protein binding moieties sites can be used to mediate binding of a vector to cell surface receptors or to serum proteins that bind to a receptor thereby increasing the efficiency of DNA transfer into the cell. Other DNA sites and structures can directly or indirectly bind to receptors in the nuclear membrane or to other proteins that go into the nucleus, thereby facilitating nuclear uptake of a vector. Other DNA sequences can directly or indirectly affect the efficiency of integration.

Suitable gene therapy vectors may, or may not, have an origin of

replication. For example, it is useful to include an origin of replication in a vector for propagation of the vector prior to administration to a patient. However, the origin of replication can often be removed before administration if the vector is designed to integrate into host chromosomal DNA or bind to host mRNA or DNA. In some situations (e.g., tumor cells) it may not be necessary for the exogenous DNA to stably integrate into the transduced cell, because transient expression may suffice to kill the tumor cells.

As noted, the present invention also provides methods and reagents for gene replacement therapy (i.e., replacement by homologous recombination of an endogenous hTERT gene with a recombinant gene). Vectors specifically designed for integration by homologous recombination may be used. Important factors for optimizing homologous recombination include the degree of sequence identity and length of homology to chromosomal sequences. The specific sequence mediating homologous recombination is also important, since integration occurs much more easily in transcriptionally active DNA. Methods and materials for constructing homologous targeting constructs are described by e.g., Mansour et al., 1988, *Nature* 336: 348; Bradley et al., 1992, *Bio/Technology* 10: 534. See also, U.S. Patent Nos. 5,627,059; 5,487,992; 5,631,153; and 5,464,764. In one embodiment, gene replacement therapy involves altering or replacing all or a portion of the regulatory sequences controlling expression of the hTERT gene that is to be regulated. For example, the hTERT promoter sequences (e.g., such as are found in SEQ. ID. NO. 6) may be disrupted (to decrease hTERT expression or to abolish a transcriptional control site) or an exogenous promoter (e.g., to increase hTERT expression) substituted.

The invention also provides methods and reagents for hTERT "gene knockout" (i.e., deletion or disruption by homologous recombination of an endogenous hTERT gene using a recombinantly produced vector). In gene knockout, the targeted sequences can be regulatory sequences (e.g., the hTERT promoter), or RNA or protein coding sequences. The use of homologous recombination to alter expression of endogenous genes is described in detail in U.S. Patent No. 5,272,071 (and the U.S. Patents cited *supra*), WO 91/09955, WO 93/09222, WO 96/29411, WO 95/31560, and

WO 91/12650. See also, Moynahan et al., 1996, *Hum. Mol. Genet.* 5:875.

The invention further provides methods for specifically killing telomerase-positive cells, or preventing transformation of telomerase negative cells to a telomerase positive state, using the hTERT gene promoter to regulate expression of a protein toxic to the cell. As shown in **Example 14**, an hTERT promoter sequence may be operably linked to a reporter gene such that activation of the promoter results in expression of the protein encoded by the reporter gene. If, instead of a reporter protein, the encoded protein is toxic to the cell, activation of the promoter leads to cell morbidity or death. In one embodiment of the present invention, a vector comprising an hTERT promoter operably linked to a gene encoding a toxic protein is introduced into cells, such as human cells, e.g., cells in a human patient, resulting in cell death of cells in which hTERT promoter activating factors are expressed, such as cancer cells. In a related embodiment, the encoded protein is not itself toxic to a cell, but encodes an activity that renders the cell sensitive to an otherwise nontoxic drug. For example, tumors can be treated by introducing a hTERT-promoter-Herpes thymidine kinase (TK) gene fusion construct into tumor cells, and administering gancyclovir or the equivalent (see, e.g., Moolton and Wells, 1990, *J. Nat'l Canc. Inst.* 82:297). The art knows of numerous other suitable toxic or potentially toxic proteins and systems (using promoter sequences other than hTERT) that may be modified and applied in accordance with the present invention by one of skill in the art upon review of this disclosure.

Gene therapy vectors may be introduced into cells or tissues *in vivo*, *in vitro* or *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into the same patient (see, e.g., U.S. Patent Nos. 5,399,493 and 5,437,994, the disclosures of which are herein incorporated by reference). Cells that can be targeted for hTERT gene therapy aimed at increasing the telomerase activity of a target cell include, but are not limited to, embryonic stem or germ cells, particularly primate or human cells, as noted *supra*, hematopoietic stem cells (AIDS and post-chemotherapy), vascular endothelial cells (cardiac and cerebral vascular disease), skin fibroblasts and basal skin keratinocytes (wound healing and burns), chondrocytes (arthritis), brain astrocytes and microglial

cells (Alzheimer's Disease), osteoblasts (osteoporosis), retinal cells (eye diseases), and pancreatic islet cells (Type I diabetes) and any of the cells listed in **Table 3**, *infra*.

In one embodiment of the invention, an inducible promoter operably linked to a TRT, such as hTRT, coding sequence (or variant) is used to modulate the proliferative capacity of cells *in vivo* or *in vitro*. In a particular embodiment, for example, insulin-producing pancreatic cells transfected with an hTRT expression vector under the control of an inducible promoter are introduced into a patient. The proliferative capacity of the cells can then be controlled by administration to the patient of the promoter activating agent (e.g., tetracycline) to enable the cells to multiply more than otherwise would have been possible. Cell proliferation can then be terminated, continued, or reinitiated as desired by the treating physician.

#### 4) VACCINES AND ANTIBODIES

Immuogenic peptides or polypeptides having an hTRT sequence can be used to elicit an anti-hTRT immune response in a patient (i.e., act as a vaccine). Exemplary immunogenic hTRT peptides and polypeptides are described *infra* in **Examples 6 and 8**. An immune response can also be raised by delivery of plasmid vectors encoding the polypeptide of interest (i.e., administration of "naked DNA"). The nucleic acids of interest can be delivered by injection, liposomes, or other means of administration. In one embodiment, immunization modes that elicit in the subject a Class I MHC restricted cytotoxic lymphocyte response against telomerase expressing cells are chosen. Once immunized, the individual or animal will elicit a heightened immune response against cells expressing high levels of telomerase (e.g., malignant cells).

Anti-hTRT antibodies, e.g., murine, human, or humanized monoclonal antibodies may also be administered to a patient (e.g., passive immunization) to effect an immune response against telomerase-expressing cells.

#### F) PHARMACEUTICAL COMPOSITIONS

In related aspects, the invention provides pharmaceutical compositions

that comprise hTERT oligo- and poly-nucleotides, polypeptides, and antibodies, agonists, antagonists, or inhibitors, alone or in combination with at least one other agent, such as a stabilizing compound, diluent, carrier, or another active ingredient or agent.

5                   The therapeutic agents of the invention may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. Any of these molecules can be administered to a patient alone, or in combination with other agents, drugs or hormones, in pharmaceutical compositions where it is mixed with suitable excipient(s), adjuvants, and/or pharmaceutically acceptable carriers. In one embodiment of the present invention, the pharmaceutically acceptable carrier is pharmaceutically inert.

10                   Administration of pharmaceutical compositions is accomplished orally or parenterally. Methods of parenteral delivery include topical, intra-arterial (*e.g.*, directly to the tumor), intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and other compounds that facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of "REMINGTON'S PHARMACEUTICAL SCIENCES" (Maack Publishing Co, Easton PA).

15                   Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, etc., suitable for ingestion by the patient. See PCT publication WO 93/23572.

25                   Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable additional

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compounds, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers include, but are not limited to sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; as well as proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound (*i.e.*, dosage).

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations for parenteral administration include aqueous solutions of active compounds. For injection, the pharmaceutical compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl

oleate or triglycerides, or liposomes. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

5 For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner similar to that known in the art (*e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, 10 encapsulating, entrapping or lyophilizing processes).

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. In other cases, the 15 preferred preparation may be a lyophilized powder in 1 mM-50 mM histidine, 0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions comprising a compound of the invention formulated in a acceptable carrier have been prepared, they can be placed in 20 an appropriate container and labeled for treatment of an indicated condition. For administration of human telomerase proteins and nucleic acids, such labeling would include amount, frequency and method of administration.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective 25 amount to achieve the intended purpose. "Therapeutically effective amount" or "pharmacologically effective amount" are well recognized phrases and refer to that amount of an agent effective to produce the intended pharmacological result. Thus, a therapeutically effective amount is an amount sufficient to ameliorate the symptoms of the disease being treated. One useful assay in ascertaining an effective amount for a 30 given application (*e.g.*, a therapeutically effective amount) is measuring the effect on

telomerase activity in a target cell. The amount actually administered will be dependent upon the individual to which treatment is to be applied, and will preferably be an optimized amount such that the desired effect is achieved without significant side-effects. The determination of a therapeutically effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in any appropriate animal model. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective amount refers to that amount of protein, polypeptide, peptide, antibody, oligo- or polynucleotide, agonist or antagonists which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals (*e.g.*,  $ED_{50}$ , the dose therapeutically effective in 50% of the population; and  $LD_{50}$ , the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio,  $ED_{50}/LD_{50}$ . Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state (*e.g.*, tumor size and location; age, weight and gender of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy). Long acting pharmaceutical compositions might be administered every 3 to 4



days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation. Guidance as to particular dosages and methods of delivery is provided in the literature (See, US Patent Nos. 4,657,760; 5,206,344; and 5,225,212, herein incorporated by reference). Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, and the like.

#### **VIII. INCREASING PROLIFERATIVE CAPACITY AND PRODUCTION OF IMMORTALIZED CELLS, CELL LINES, AND ANIMALS**

As discussed above, most vertebrate cells senesce after a finite number of divisions in culture (e.g., 50 to 100 divisions). Certain variant cells, however, are able to divide indefinitely in culture (e.g., HeLa cells, 293 cells) and, for this reason, are useful for research and industrial applications. Usually these immortal cell lines are derived from spontaneously arising tumors, or by transformation by exposure to radiation or a tumor-inducing virus or chemical. Unfortunately, a limited selection of cell lines, especially human cell lines representing differentiated cell function, is available. Moreover, the immortal cell lines presently available are characterized by chromosomal abnormalities (e.g., aneuploidy, gene rearrangements, or mutations). Further, many long-established cell lines are relatively undifferentiated (e.g., they do not produce highly specialized products of the sort that uniquely characterize particular tissues or organs). Thus, there is a need for new methods of generating immortal cells, especially human cells. One use for immortalized cells is in production of natural proteins and recombinant proteins (e.g., therapeutic polypeptides), or antibodies, for which a stable, genetically normal cell line is preferred. For production of some recombinant proteins, specialized cell types may also be preferred (e.g., pancreatic cells for the production of human insulin). Another use for immortalized cells is for introduction into a patient for gene therapy, or for replacement of diseased or damaged cells or tissue. For example, autologous immune cells containing or expressing a, e.g., recombinant hTERT gene or polypeptide of the invention may be used for cell

replacement in a patient after aggressive cancer therapy, e.g., whole body irradiation. Another use for immortalized cells is for *ex vivo* production of “artificial” tissues or organs (e.g., skin) for therapeutic use. Another use for such cells is for screening or validation of drugs, such as telomerase-inhibiting drugs, or for use in production of vaccines. Additional uses of the cells of the invention will be apparent to those of skill.

The immortalized cells and cell lines as well as those of merely increased replicative capacity, of the invention are made by increasing telomerase activity in the cell. Any method disclosed herein for increasing telomerase activity may be used. Thus, in one embodiment, cells are immortalized by increasing the amount of an hTERT polypeptide in the cell. In one embodiment, hTERT levels are increased by introducing an hTERT expression vector into the cell (with stable transfection sometimes preferred). As discussed above, the hTERT coding sequence is usually operably linked to a promoter, which may be inducible or constitutively active in the cell.

In one embodiment, a polynucleotide comprising a sequence encoding a polypeptide of **SEQ. ID. NO: 2**, which sequence is operably linked to a promoter (e.g., a constitutively expressed promoter, e.g., a sequence of **SEQ. ID. NO: 6**), is introduced into the cell. In one embodiment the polynucleotide comprises a sequence of **SEQ. ID. NO: 1**. Preferably the polynucleotide includes polyadenylation and termination signals. In other embodiments, additional elements such as enhancers or others discussed *supra* are included. In an alternative embodiment, the polynucleotide does not include a promoter sequence, such sequence being provided by the target cell endogenous genome following integration (e.g., recombination, e.g., homologous recombination) of the introduced polynucleotide. The polynucleotide may be introduced into the target cell by any method, including any method disclosed herein, such as lipofection, electroporation, virosomes, liposomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA).

Using the methods of the invention, any vertebrate cell can be caused to have an increased proliferative capacity or even be immortalized and sustained indefinitely in culture. In one embodiment the cells are mammalian, with human cells preferred for many applications. Examples of human cells that can be immortalized

include those listed in **Table 3**.

It will be recognized that the "diagnostic" assays of the invention described *infra* may be used to identify and characterize the immortalized cells of the invention.

5

**TABLE 3**

**HUMAN CELLS IN WHICH HTRT EXPRESSION MAY BE INCREASED**

**Keratinizing Epithelial Cells**

- keratinocyte of epidermis (differentiating epidermal cell)
- 10 basal cell of epidermis (stem cell)
- keratinocyte of fingernails and toenails
- basal cell of nail bed (stem cell)
- hair shaft cells
  - medullary, cortical, cuticular; hair-root sheath
  - 15 cells, cuticular, of Huxley's layer, of Henle's layer
  - external; hair matrix cell (stem cell)

**Cells of Wet Stratified Barrier Epithelia**

- surface epithelial cell of stratified squamous epithelium
- 20 of tongue, oral cavity, esophagus, anal canal, distal
- urethra, vagina
- basal cell of these epithelia (stem cell)
- cell of external corneal epithelium
- cell of urinary epithelium (lining bladder and urinary
- 25 ducts)

**Epithelial Cells Specialized for Exocrine Secretion**

- cells of salivary gland
  - mucous cell (secretion rich in polysaccharide)
  - 30 serous cell (secretion rich in glycoprotein enzymes)
  - cell of von Ebner's gland in tongue (secretion to
  - wash over taste buds)
- cell of mammary gland, secreting milk
- cell of lacrimal gland, secreting tears
- 35 cell of ceruminous gland of ear, secreting wax
- cell of eccrine sweat gland, secreting glycoproteins (dark
- cell)
- cell of eccrine sweat gland, secreting small molecules
- (clear cell)
- 40 cell of apocrine sweat gland (odoriferous secretion,
- sex-hormone sensitive)

- cell of gland of Moll in eyelid (specialized sweat gland)  
cell of sebaceous gland, secreting lipid-rich sebum  
cell of Bowman's gland in nose (secretion to wash over  
olfactory epithelium)
- 5 cell of Brunner's gland in duodenum, secreting alkaline  
solution of mucus and enzymes  
cell of seminal vesicle, secreting components of seminal  
fluid, including fructose (as fuel for swimming sperm)  
cell of prostate gland, secreting other components of  
10 seminal fluid  
cell of bulbourethral gland, secreting mucus  
cell of Bartholin's gland, secreting vaginal lubricant  
cell of gland of Littre, secreting mucus  
cell of endometrium of uterus, secreting mainly  
15 carbohydrates  
isolated goblet cell of respiratory and digestive tracts,  
secreting mucus  
mucous cell of lining of stomach  
zymogenic cell of gastric gland, secreting pepsinogen  
20 oxyntic cell of gastric gland, secreting HCl  
acinar cell of pancreas, secreting digestive enzymes and  
bicarbonate  
Paneth cell of small intestine, secreting lysozyme  
type II pneumocyte of lung, secreting surfactant  
25 Clara cell of lung

**Cells specialized for Secretion of Hormones**

- cells of anterior pituitary, secreting  
growth hormone, follicle-stimulating hormone,  
30 luteinizing hormone, prolactin, adrenocorticotrophic  
hormone, and thyroid-stimulating hormone,  
cell of intermediate pituitary, secreting  
melanocyte-stimulating hormone  
cells of posterior pituitary, secreting  
35 oxytocin, vasopressin  
cells of gut, secreting  
serotonin, endorphin, somatostatin, gastrin,  
secretin, cholecystokinin, insulin and glucagon  
cells of thyroid gland, secreting  
40 thyroid hormone, calcitonin  
cells of parathyroid gland, secreting  
parathyroid hormone, oxyphil cell  
cells of adrenal gland, secreting  
epinephrine, norepinephrine, and steroid hormones;

- mineralocorticoids  
glucocorticoids  
cells of gonads, secreting  
testosterone (Leydig cell of testis)  
5 estrogen (theca interna cell of ovarian follicle)  
progesterone (corpus luteum cell of ruptured ovarian  
follicle)  
cells of juxtaglomerular apparatus of kidney  
juxtaglomerular cell (secreting renin)  
10 macula densa cell  
peripolar cell  
mesangial cell
- Epithelial Absorptive Cells in Gut, Exocrine Glands, and  
15 Urogenital Tract**  
brush border cell of intestine (with microvilli)  
striated duct cell of exocrine glands  
gall bladder epithelial cell  
brush border cell of proximal tubule of kidney  
20 distal tubule cell of kidney  
nonciliated cell of ductulus efferens  
epididymal principal cell  
epididymal basal cell
- 25 Cells Specialized for Metabolism and Storage**  
hepatocyte (liver cell)  
fat cells  
white fat  
brown fat  
30 lipocyte of liver
- Epithelial Cells Serving Primarily a Barrier Function,  
Lining the Lung, Gut, Exocrine Glands, and Urogenital  
Tract**  
35 type I pneumocyte (lining air space of lung)  
pancreatic duct cell (centroacinar cell)  
nonstriated duct cell of sweat gland, salivary gland,  
mammary gland  
parietal cell of kidney glomerulus  
40 podocyte of kidney glomerulus  
cell of thin segment of loop of Henle (in kidney)  
collecting duct cell (in kidney)  
duct cell of seminal vesicle, prostate gland

**Epithelial Cells Lining Closed Internal Body Cavities**

vascular endothelial cells of blood vessels and lymphatics

fenestrated

continuous

5 splenic

synovial cell (lining joint cavities, secreting largely  
hyaluronic acid)

serosal cell (lining peritoneal, pleural, and pericardial  
cavities)

10 squamous cell lining perilymphatic space of ear

cells lining endolymphatic space of ear

squamous cell

columnar cells of endolymphatic sac

with microvilli

15 without microvilli

"dark" cell

vestibular membrane cell (resembling choroid plexus  
cell)

stria vascularis basal cell

20 stria vascularis marginal cell

cell of Claudius

cell of Boettcher

choroid plexus cell (secreting cerebrospinal fluid)

squamous cell of pia-arachnoid

25 cells of ciliary epithelium of eye

pigmented

nonpigmented

corneal "endothelial" cell

30 **Ciliated Cells with Propulsive Function**

of respiratory tract

of oviduct and of endometrium of uterus (in female)

of rete testis and ductulus efferens (in male)

of central nervous system (ependymal cell lining brain

35 cavities)

**Cells Specialized for Secretion of Extracellular Matrix**

epithelial:

ameloblast (secreting enamel of tooth)

40 planum semilunatum cell of vestibular apparatus of ear  
(secreting proteoglycan)

interdental cell of organ of Corti (secreting tectorial

"membrane" covering hair cells of organ of Corti)

nonepithelial (connective tissue)

fibroblasts (various-of loose connective tissue, of cornea, of tendon, of reticular tissue of bone marrow, etc.)

- pericyte of blood capillary
- 5 nucleus pulposus cell of intervertebral disc
- cementoblast/cementocyte (secreting bonelike cementum of root of tooth)
- odontoblast/odontocyte (secreting dentin of tooth)
- chondrocytes
- 10 of hyaline cartilage, of fibrocartilage, of elastic cartilage
- osteoblast/osteocyte
- osteoprogenitor cell (stem cell of osteoblasts)
- hyalocyte of vitreous body of eye
- 15 stellate cell of perilymphatic space of ear

**Contractile Cells**

- skeletal muscle cells
- red (slow)
- 20 white (fast)
- intermediate
- muscle spindle—nuclear bag
- muscle spindle—nuclear chain
- satellite cell (stem cell)
- 25 heart muscle cells
- ordinary
- nodal
- Purkinje fiber
- smooth muscle cells
- 30 myoepithelial cells
- of iris
- of exocrine glands

**Cells of Blood and Immune System**

- 35 red blood cell
- megakaryocyte
- macrophages
- monocyte
- connective tissue macrophage (various)
- 40 Langerhans cell (in epidermis)
- osteoclast (in bone)
- dendritic cell (in lymphoid tissues)
- microglial cell (in central nervous system)
- neutrophil

eosinophil  
basophil  
mast cell  
T lymphocyte  
5     helper T cell  
       suppressor T cell  
       killer T cell  
B lymphocyte  
   IgM  
10     IgG  
       IgA  
       IgE  
killer cell  
stem cells for the blood and immune system (various)  
15  
**Sensory Transducers**  
photoreceptors  
   rod  
   cones  
20     blue sensitive  
       green sensitive  
       red sensitive  
hearing  
   inner hair cell of organ of Corti  
25     outer hair cell of organ of Corti  
acceleration and gravity  
   type I hair cell of vestibular apparatus of ear  
   type II hair cell of vestibular apparatus of ear  
taste  
30     type II taste bud cell  
smell  
   olfactory neuron  
   basal cell of olfactory epithelium (stem cell for  
   olfactory  
35     neurons)  
blood Ph  
   carotid body cell  
       type I  
       type II  
40 touch  
   Merkel cell of epidermis  
   primary sensory neurons specialized for touch  
temperature  
   primary sensory neurons specialized for temperature



cold sensitive  
heat sensitive

pain

- primary sensory neurons specialized for pain
- 5 configurations and forces in musculoskeletal system
- proprioceptive primary sensory neurons

**Autonomic Neurons**

- cholinergic
- 10 adrenergic
- peptidergic

**Supporting Cells of Sense Organs and of Peripheral Neurons**

- supporting cells of organ of Corti
- 15 inner pillar cell
- outer pillar cell
- inner phalangeal cell
- outer phalangeal cell
- border cell
- 20 Hensen cell
- supporting cell of vestibular apparatus
- supporting cell of taste bud (type I taste bud cell)
- supporting cell of olfactory epithelium
- Schwann cell
- 25 satellite cell (encapsulating peripheral nerve cell
- bodies)
- enteric glial cell

**Neurons and Glial Cells of Central Nervous System**

- 30 neurons
- glial cells
- astrocyte
- oligodendrocyte

**35 Lens Cells**

- anterior lens epithelial cell
- lens fiber (crystallin-containing cell)

**Pigment Cells**

melanocyte  
retinal pigmented epithelial cell

5    **Germ Cells**

oogonium/oocyte  
spermatocyte  
spermatogonium (stem cell for spermatocyte)

10   **Nurse Cells**

ovarian follicle cell  
Sertoli cell (in testis)  
thymus epithelial cell

15   **Stem Cells**

embryonic stem cell  
embryonic germ cell  
    adult stem cell  
    fetal stem cell

20

**IX. DIAGNOSTIC ASSAYS**

**A) INTRODUCTION**

**1) TRT ASSAYS**

25            The present invention provides a wide variety of assays for TRT, preferably hTRT, and telomerase. These assays provide, *inter alia*, the basis for sensitive, inexpensive, convenient, and widely applicable assays for diagnosis and prognosis of a number of human diseases, of which cancer is an illustrative example. As noted *supra*, hTRT gene products (protein and mRNA) are usually elevated in

30   immortal human cells relative to most normal mortal cells (i.e., telomerase-negative cells and most telomerase-positive normal adult somatic cells). Thus, in one aspect, the invention provides assays useful for detecting or measuring the presence, absence, or quantity of an hTRT gene product in a sample from, or containing, human or other mammalian or eukaryotic cells to characterize the cells as immortal (such as a malignant

35   tumor cell) or mortal (such as most normal somatic cells in adults) or as telomerase positive or negative.

Any condition characterized by the presence or absence of an hTERT gene product (i.e., protein or RNA) may be diagnosed using the methods and materials described herein. These include, as described more fully below, cancers, other diseases of accelerated cell proliferation, immunological disorders, fertility, infertility, and others. Moreover, because the degree to which telomerase activity is elevated in cancer cells is correlated with characteristics of the tumor, such as metastatic potential, monitoring hTERT, mRNA or protein levels can be used to estimate and predict the likely future progression of a tumor.

In one aspect, the diagnostic and prognostic methods of the invention entail determining whether a human TRT gene product is present in a biological sample (e.g., from a patient). In a second aspect, the abundance of hTERT gene product in a biological sample (e.g., from a patient) is determined and compared to the abundance in a control sample (e.g., normal cells or tissues). In a third aspect, the cellular or intracellular localization of a hTERT gene product is determined in a cell or tissue sample. In a fourth aspect, host (e.g., patient) cells are assayed to identify nucleic acids with sequences characteristic of a heritable propensity for abnormal hTERT gene expression (abnormal quantity, regulation, or product), such as is useful in genetic screening or genetic counseling. In a fifth aspect, the assays of the invention are used to detect the presence of anti-hTERT antibodies (e.g., in patient serum). The methods described below in some detail are indicative of useful assays that can be carried out using the sequences and relationships disclosed herein. However, numerous variations or other applications of these assays will be apparent to those of ordinary skill in the art.

It will be recognized that, although the assays below are presented in terms of diagnostic and prognostic methods, they may be used whenever an hTERT gene, gene product, or variant is to be detected, quantified, or characterized. Thus, for example, the "diagnostic" methods described *infra* are useful for assays of hTERT or telomerase during production and purification of hTERT or human telomerase, for characterization of cell lines derived from human cells (e.g., to identify immortal lines), for characterization of cells, non-human animals, plants, fungi, bacteria or other organisms that comprise a human TRT gene or gene product (or fragments thereof).

As used herein, the term "diagnostic" has its usual meaning of identifying the presence or nature of a disease (e.g., cancer), condition (e.g., infertile, activated), or status (e.g., fertile), and the term "prognostic" has its usual meaning of predicting the probable development and/or outcome of a disease or condition.

- 5 Although these two terms are used in somewhat different ways in a clinical setting, it will be understood that any of the assays or assay formats disclosed below in reference to "diagnosis" are equally suitable for determination of prognosis because it is well established that higher telomerase activity levels are associated with poorer prognoses for cancer patients, and because the present invention provides detection methods
- 10 specific for hTERT, which is expressed at levels that closely correlate with telomerase activity in a cell .

## 2) DIAGNOSIS AND PROGNOSIS OF CANCER

The determination of an hTERT gene, mRNA or protein level above normal or standard range is indicative of the presence of telomerase-positive cells, or immortal, of which certain tumor cells are examples. Because certain embryonic and fetal cells, as well as certain adult stem cells, express telomerase, the present invention also provides methods for determining other conditions, such as pregnancy, by the detection or isolation of telomerase positive fetal cells from maternal blood. These values can be used to make, or aid in making, a diagnosis, even when the cells would not have been classified as cancerous or otherwise detected or classified using traditional methods. Thus, the methods of the present invention permit detection or verification of cancerous or other conditions associated with telomerase with increased confidence, and possibly at an earlier stage. The assays of the invention allow discrimination between different classes and grades of human tumors or other cell-proliferative diseases by providing quantitative assays for the hTERT gene and gene products and thereby facilitate the selection of appropriate treatment regimens and accurate diagnoses. Moreover, because levels of telomerase activity can be used to distinguish between benign and malignant tumors (e.g., U.S. Patent No. 5,489,508; Hiyama et al., 1997, *Proc. Am Ass. Cancer Res.* 38:637), to predict immanence of invasion (e.g., U.S. Patent No. 5,639,613; Yashima et al., 1997, *Proc. Am Ass. Cancer Res.* 38:326), and to correlate with metastatic potential (e.g., U.S. Patent No. 5,648,215; Pandita et al, 1996, *Proc. Am Ass. Cancer Res.* 37:559), these assays will be useful for prophylaxis, detection, and treatment of a wide variety of human cancers.

For prognosis of cancers (or other diseases or conditions characterized by elevated telomerase), a prognostic value of hTERT gene product (mRNA or protein) or activity for a particular tumor type, class or grade, is determined as described *infra*. hTERT protein or mRNA levels or telomerase activity in a patient can also be determined (e.g., using the assays disclosed herein) and compared to the prognostic level.

Depending on the assay used, in some cases the abundance of an hTERT gene product in a sample will be considered elevated whenever it is detectable by the

assay. Due to the low abundance of hTERT mRNA and protein even in telomerase-positive cells, and the rarity or non-existence of these gene products in normal or telomerase-negative cells, sensitive assays are required to detect the hTERT gene product if present at all in normal cells. If less sensitive assays are selected, hTERT gene products will be undetectable in healthy tissue but will be detectable in telomerase-positive cancer or other telomerase-positive cells. Typically, the amount of hTERT gene product in an elevated sample is at least about five, frequently at least about ten, more often at least about 50, and very often at least about 100 to 1000 times higher than the levels in telomerase-negative control cells or cells from healthy tissues in an adult, where the percentage of telomerase-positive normal cells is very low.

The diagnostic and prognostic methods of the present invention can be employed with any cell or tissue type of any origin and can be used to detect an immortal or neoplastic cell, or tumor tissue, or cancer, of any origin. Types of cancer that may be detected include, but are not limited to, all those listed *supra* in the discussion of therapeutic applications of hTERT.

The assays of the invention are also useful for monitoring the efficacy of therapeutic intervention in patients being treated with anticancer regimens. Anticancer regimens that can be monitored include all presently approved treatments (including chemotherapy, radiation therapy, and surgery) and also includes treatments to be approved in the future, such as telomerase inhibition or activation therapies as described herein. (See, e.g., See PCT Publication Nos. 96/01835 and 96/40868 and U.S. Patent No. 5,583,016; see also U.S. patent application Serial Nos. 08/472,802 and 08/482,115, both filed 7 June 1995; 08/521,634, filed 31 Aug 95; 08/714,482, filed 16 Sep 96; and 08/770,564 and 08/770,565, both filed 20 December 1996, all of which are incorporated by reference in their entirety).

In another aspect, the assays described below are useful for detecting certain variations in hTERT gene sequence (mutations and heritable hTERT alleles) that are indicative of a predilection for cancers or other conditions associated with abnormal regulation of telomerase activity (infertility, premature aging).

### 3) DIAGNOSIS OF CONDITIONS OTHER THAN CANCER

In addition to diagnosis of cancers, the assays of the present invention have numerous other applications. The present invention provides reagents and methods/diagnosis of conditions or diseases characterized by under- or over-expression of telomerase or hTERT gene products in cells. In adults, a low level of telomerase activity is normally found in a limited complement of normal human somatic cells, e.g., stem cells, activated lymphocytes and germ cells, and is absent from other somatic cells. Thus, the detection of hTERT or telomerase activity in cells in which it is normally absent or inactive, or detection at abnormal (i.e., higher or lower than normal) levels in cells in which hTERT is normally present at a low level (such as stem cells, activated lymphocytes and germ cells), may be diagnostic of a telomerase-related disease or condition or may be used to identify or isolate specific cell type. Examples of such diseases and conditions include: diseases of cell proliferation, immunological disorders, infertility, diseases of immune cell function, pregnancy, fetal abnormalities, premature aging, and others. Moreover, the assays of the invention are useful for monitoring the effectiveness of therapeutic intervention (including but not limited to drugs that modulate telomerase activity) in a patient or in a cell- or animal-based assay.

In one aspect, the invention provides assays useful for diagnosing infertility. Human germ cells (e.g., spermatogonia cells, their progenitors or descendants) are capable of indefinite proliferation and characterized by high telomerase activity. Abnormal levels or products or diminished levels of hTERT gene products can result in inadequate or abnormal production of spermatozoa, leading to infertility or disorders of reproduction. Accordingly, the invention provides assays (methods and reagents) for diagnosis and treatment of "telomerase-based" reproductive disorders. Similarly, the assays can be used to monitor the efficacy of contraceptives (e.g., male contraceptives) that target or indirectly affect sperm production (and which would reduce hTERT levels or telomerase activity).

In another aspect, the invention provides assays for analysis of telomerase and hTERT levels and function in stem cells, fetal cells, embryonic cells, activated lymphocytes and hematopoietic stem cells. For example, assays for hTERT

gene product detection can be used to monitor immune function generally (e.g., by monitoring the prevalence of activated lymphocytes or abundance of progenitor stem cells), to identify or select or isolate activated lymphocytes or stem cells (based on elevated hTERT levels), and to monitor the efficacy of therapeutic interventions targeting these tissues (e.g., immunosuppressive agents or therapeutic attempt to expand a stem cell population).

The invention also provides assays useful for identification of anti-telomerase and anti-TERT immunoglobulins (found in serum from a patient). The materials and assays described herein can be used to identify patients in which such autoimmune antibodies are found, permitting diagnosis and treatment of the condition associated with the immunoglobulins.

#### **4) MONITORING CELLS IN CULTURE**

The assays described herein are also useful for monitoring the expression of hTERT gene products and characterization of hTERT genes in cells *ex vivo* or *in vitro*. Because elevated hTERT levels are characteristic of immortalized cells, the assays of the invention can be used, for example, to screen for, or identify, immortalized cells or to identify an agent capable of mortalizing immortalized cells by inhibiting hTERT expression or function. For example, the assay will be useful for identifying cells immortalized by increased expression of hTERT in the cell, e.g., the expression of a recombinant hTERT, by increased expression of an endogenously coded hTERT (e.g., by promoter activation).

Similarly, these assays may be used to monitor hTERT expression in transgenic animals or cells (e.g., yeast or human cells containing a hTERT gene). In particular, the effects of certain treatments (e.g., application of known or putative telomerase antagonists) on the hTERT levels in human and nonhuman cells expressing the hTERT of the invention can be used for identifying useful drugs and drug candidates (e.g., telomerase activity-modulating drugs).

#### **B) NORMAL, DIAGNOSTIC, AND PROGNOSTIC VALUES**



Assays for the presence or quantity of hTERT gene products may be carried out and the results interpreted in a variety of ways, depending on the assay format, the nature of the sample being assayed, and the information sought. For example, the steady state abundance of hTERT gene products is so low in most human somatic tissues that they are undetectable by certain assays. Moreover, there is generally no telomerase activity in these cells, making verification of activity quite easy. Conversely, hTERT protein and/or hTERT mRNA or telomerase is sufficiently abundant in other telomerase-positive tissues, e.g., malignant tumors, so that the same can be detected using the same assays. Even in those somatic cell types in which low levels of telomerase activity can normally be detected (e.g., stem cells, and certain activated hematopoietic system cells), the levels of hTERT mRNA and telomerase activity are a small fraction (e.g., estimated at about 1% or less) of the levels in immortal cells; thus, immortal and mortal cells may be easily distinguished by the methods of the present invention. It will be appreciated that, when a "less sensitive" assay is used, the mere detection of the hTERT gene product in a biological sample can itself be diagnostic, without the requirement for additional analysis. Moreover, although the assays described below can be made exquisitely sensitive they may also, if desired, be made less sensitive (e.g., through judicious choice of buffers, wash conditions, numbers of rounds of amplification, reagents, and/or choice of signal amplifiers). Thus, virtually any assay can be designed so that it detects hTERT gene products only in biological samples in which they are present at a particular concentration, e.g. a higher concentration than in healthy or other control tissue. In this case, any detectable level of hTERT mRNA or protein will be considered elevated in cells from post-natal human somatic tissue (other than hematopoietic cells and other stem cells).

In some cases, however, it will be desirable to establish normal or baseline values (or ranges) for hTERT gene product expression levels, particularly when very sensitive assays capable of detecting very low levels of hTERT gene products that may be present in normal somatic cells are used. Normal levels of expression or normal expression products can be determined for any particular population,

subpopulation, or group of organisms according to standard methods well known to those of skill in the art. Generally, baseline (normal) levels of hTERT protein or hTERT mRNA are determined by quantitating the amount of hTERT protein and/or mRNA in biological samples (e.g., fluids, cells or tissues) obtained from normal (healthy) subjects, e.g., a human subject. For certain samples and purposes, one may desire to quantitate the amount of hTERT gene product on a per cell, or per tumor cell, basis. To determine the cellularity of a sample, one may measure the level of a constitutively expressed gene product or other gene product expressed at known levels in cells of the type from which the sample was taken. Alternatively, normal values of hTERT protein or hTERT mRNA can be determined by quantitating the amount of hTERT protein/RNA in cells or tissues known to be healthy, which are obtained from the same patient from whom diseased (or possibly diseased) cells are collected or from a healthy individual. Alternatively, baseline levels can be defined in some cases as the level present in non-immortal human somatic cells in culture. It is possible that normal (baseline) values may differ somewhat between different cell types (for example, hTERT mRNA levels will be higher in testis than kidney), or according to the age, sex, or physical condition of a patient. Thus, for example, when an assay is used to determine changes in hTERT levels associated with cancer, the cells used to determine the normal range of hTERT gene product expression may be cells from persons of the same or a different age, depending on the nature of the inquiry. Application of standard statistical methods used in molecular genetics permits determination of baseline levels of expression, as well as significant deviations from such baseline levels.

In carrying out the diagnostic and prognostic methods of the invention, as described above, it will sometimes be useful to refer to "diagnostic" and "prognostic values." As used herein, "diagnostic value" refers to a value that is determined for the hTERT gene product detected in a sample which, when compared to a normal (or "baseline") range of the hTERT gene product is indicative of the presence of a disease. The disease may be characterized by high telomerase activity (e.g., cancer), the absence of telomerase activity (e.g., infertility), or some intermediate value. "Prognostic value" refers to an amount of the hTERT gene product detected in a given cell type (e.g.,

malignant tumor cell) that is consistent with a particular diagnosis and prognosis for the disease (e.g., cancer). The amount (including a zero amount) of the hTERT gene product detected in a sample is compared to the prognostic value for the cell such that the relative comparison of the values indicates the presence of disease or the likely outcome of the disease (e.g., cancer) progression. In one embodiment, for example, to assess tumor prognosis, data are collected to obtain a statistically significant correlation of hTERT levels with different tumor classes or grades. A predetermined range of hTERT levels is established for the same cell or tissue sample obtained from subjects having known clinical outcomes. A sufficient number of measurements is made to produce a statistically significant value (or range of values) to which a comparison will be made. The predetermined range of hTERT levels or activity for a given cell or tissue sample can then be used to determine a value or range for the level of hTERT gene product that would correlated to favorable (or less unfavorable) prognosis (e.g., a "low level" in the case of cancer). A range corresponding to a "high level" correlated to an (or a more) unfavorable prognosis in the case of cancer can similarly be determined. The level of hTERT gene product from a biological sample (e.g., a patient sample) can then be determined and compared to the low and high ranges and used to predict a clinical outcome.

Although the discussion above refers to cancer for illustration, it will be understood that diagnostic and prognostic values can also be determined for other diseases (e.g., diseases of cell proliferation) and conditions and that, for diseases or conditions other than cancer, a "high" level may be correlated with the desired outcome and a "low" level correlated with an unfavorable outcome. For example, some diseases may be characterized by a deficiency (e.g., low level) of telomerase activity in stem cells, activated lymphocytes, or germline cells. In such cases, "high" levels of hTERT gene products relative to cells of similar age and/or type (e.g., from other patients or other tissues in a particular patient) may be correlated with a favorable outcome.

It will be appreciated that the assay methods do not necessarily require measurement of absolute values of hTERT, unless it is so desired, because relative values are sufficient for many applications of the methods of the present invention. Where

quantitation is desirable, the present invention provides reagents such that virtually any known method for quantitating gene products can be used.

The assays of the invention may also be used to evaluate the efficacy of a particular therapeutic treatment regime in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. In these cases, it may be desirable to establish the baseline for the patient prior to commencing therapy and to repeat the assays one or more times through the course of treatment, usually on a regular basis, to evaluate whether hTERT levels are moving toward the desired endpoint (e.g., reduced expression of hTERT when the assay is for cancer) as a result of the treatment.

One of skill will appreciate that, in addition to the quantity or abundance of hTERT gene products, variant or abnormal expression patterns (e.g., abnormal amounts of RNA splicing variants) or variant or abnormal expression products (e.g., mutated transcripts, truncated or non-sense polypeptides) may also be identified by comparison to normal expression levels and normal expression products. In these cases determination of "normal" or "baseline" involves identifying healthy organisms and/or tissues (*i.e.* organisms and/or tissues without hTERT expression dysregulation or neoplastic growth) and measuring expression levels of the variant hTERT gene products (e.g., splicing variants), or sequencing or detecting the hTERT gene, mRNA, or reverse transcribed cDNA to obtain or detect typical (normal) sequence variations. Application of standard statistical methods used in molecular genetics permits determination of significant deviations from such baseline levels.

### **C) DETECTION AND QUANTITATION OF TERT GENE PRODUCTS**

As has been emphasized herein, hTERT gene products are usually found in most normal somatic cells at extremely low levels. For example, the mRNA encoding hTERT protein is extremely rare or absent in all telomerase-negative cell types studied thus far. In immortal cells, such as 293 cells, hTERT mRNA may be present at only about 100 copies per cell, while normal somatic cells may have as few as one or zero copies per cell. It will thus be apparent that, when highly sensitive assays for hTERT gene products are desired, it will sometimes be advantageous to incorporate

signal or target amplification technologies into the assay format. See, for example, Plenat et al., 1997, *Ann. Pathol.* 17:17 (fluoresceinyl-tyramide signal amplification); Zehbe et al., 1997, *J. Pathol.* 150:1553 (catalyzed reporter deposition); other references listed herein (e.g., for bDNA signal amplification, for PCR and other target amplification formats); and other techniques known in the art.

As noted above, it is often unnecessary to quantitate the hTERT mRNA or protein in the assays disclosed herein, because the detection of a hTERT gene product (under assay conditions in which the product is not detectable in control, e.g., telomerase-negative cells) is in itself sufficient for a diagnosis. As another example, when the levels of product found in a test (e.g., tumor) and control (e.g., healthy cell) samples are directly compared, quantitation may be superfluous.

When desired, however, quantities of hTERT gene product measured in the assays described herein may be described in a variety of ways, depending on the method of measurement and convenience. Thus, normal, diagnostic, prognostic, high or low quantities of hTERT protein/mRNA may be expressed as standard units of weight per quantity of biological sample (e.g., picograms per gram tissue, picograms per  $10^{12}$  cells), as a number of molecules per quantity of biological sample (e.g., transcripts/cell, moles/cell), as units of activity per cell or per other unit quantity, or by similar methods. The quantity of hTERT gene product can also be expressed in relation to the quantity of another molecule; examples include: number of hTERT transcripts in sample/number of 28S rRNA transcripts in sample; nanograms of hTERT protein/nanograms of total protein; and the like.

When measuring hTERT gene products in two (or more) different samples, it will sometimes be useful to have a common basis of comparison for the two samples. For example, when comparing a sample of normal tissue and a sample of cancerous tissue, equal amounts of tissue (by weight, volume, number of cells, etc.) can be compared. Alternatively, equivalents of a marker molecule (e.g., 28S rRNA, hTERT, telomerase activity, telomere length, actin) may be used. For example, the amount of hTERT protein in a healthy tissue sample containing 10 picograms of 28S rRNA can be compared to a sample of diseased tissue containing the same amount of 28S rRNA.

It will also be recognized by those of skill that virtually any of the assays described herein can be designed to be quantitative. Typically, a known quantity or source of an hTERT gene product (e.g., produced using the methods and compositions of the invention) is used to calibrate the assay.

5           In certain embodiments, assay formats are chosen that detect the presence, absence, or abundance of a hTERT allele or gene product in each cell in a sample (or in a representative sampling). Examples of such formats include those that detect a signal by histology (e.g., immunohistochemistry with signal-enhancing or target-enhancing amplification steps) or fluorescence-activated cell analysis or cell  
10       sorting (FACS)). These formats are particularly advantageous when dealing with a highly heterogeneous cell population (e.g., containing multiple cells types in which only one or a few types have elevated hTERT levels, or a population of similar cells expressing telomerase at different levels).

#### 15           **D) SAMPLE COLLECTION**

          The hTERT gene or gene product (i.e., mRNA or polypeptide) is preferably detected and/or quantified in a biological sample. Such samples include, but are not limited to, cells, (including whole cells, cell fractions, cell extracts, and cultured cells or cell lines), tissues (including blood, blood cells (e.g., white cells), tissue  
20       samples such as fine needle biopsy samples (e.g., from prostate, breast, thyroid, *etc.*), body fluids (e.g., urine, sputum, amniotic fluid, blood, peritoneal fluid, pleural fluid, semen) or cells collected therefrom (e.g., bladder cells from urine, lymphocytes from blood), media (from cultured cells or cell lines), and washes (e.g., of bladder and lung). Biological samples may also include sections of tissues such as frozen sections taken  
25       for histological purposes. For cancer diagnosis and prognosis, a sample will be obtained from a cancerous or precancerous or suspected cancerous tissue or tumor. It will sometimes be desirable to freeze a biological sample for later analysis (e.g., when monitoring efficacy of drug treatments).

          In some cases, the cells or tissues may be fractionated before analysis.  
30       For example, in a tissue biopsy from a patient, a cell sorter (e.g., a fluorescence-

activated cell sorter) may be used to sort cells according to characteristics such as expression of a surface antigen (e.g., a tumor specific antigen) according to well known methods.

5 Although the sample is typically taken from a human patient or cell line, the assays can be used to detect hTRT homolog genes or gene products in samples from other animals. Alternatively, hTRT genes and gene products can be assayed in transgenic animals or organisms expressing a human TRT protein or nucleic acid sequence.

10 The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris-buffer, or the like, at physiological pH can be used.

15 A "biological sample" obtained from a patient can be referred to either as a "biological sample" or a "patient sample." It will be appreciated that analysis of a "patient sample" need not necessarily require removal of cells or tissue from the patient. For example, appropriately labeled hTRT-binding agents (e.g., antibodies or nucleic acids) can be injected into a patient and visualized (when bound to the target) using standard imaging technology (e.g., CAT, NMR, and the like.)

## 20 **E) NUCLEIC ACID ASSAYS**

In one embodiment, this invention provides for methods of detecting and/or quantifying expression of hTRT mRNAs (including splicing or sequence variants and alternative alleles). In an alternative embodiment, the invention provides methods for detecting and analyzing normal or abnormal hTRT genes (or a fragments thereof). The form of such qualitative or quantitative assays may include, but is not  
25 limited to, amplification-based assays with or without signal amplification, hybridization based assays, and combination amplification-hybridization assays. It will be appreciated by those of skill that the distinction between hybridization and amplification is for convenience only: as illustrated in the examples below, many assay  
30 formats involve elements of both hybridization and amplification, so that the

categorization is somewhat arbitrary in some cases.

### 1) PREPARATION OF NUCLEIC ACIDS

In some embodiments, nucleic acid assays are performed with a sample of nucleic acid isolated from the cell, tissue, organism, or cell line to be tested. The nucleic acid (*e.g.*, genomic DNA, RNA or cDNA) may be "isolated" from the sample according to any of a number of methods well known to those of skill in the art. In this context, "isolated" refers to any separation of the species or target to be detected from any other substance in the mixture, but does not necessarily indicate a significant degree of purification of the target. One of skill will appreciate that, where alterations in the copy number of the hTERT gene are to be detected, genomic DNA is the target to be detected. Conversely, where expression levels of a gene or genes are to be detected, RNA is the target to be detected in a nucleic acid-based assay. In one preferred embodiment, the nucleic acid sample is the total mRNA (*i.e.*, poly(A)<sup>+</sup> RNA) in a biological sample. Methods for isolating nucleic acids are well known to those of skill in the art and are described, for example, Tijssen, P. ed. of LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, PART I. THEORY AND NUCLEIC ACID PREPARATION, Elsevier, N.Y. (1993) Chapt. 3, which is incorporated herein by reference. In one embodiment, the total nucleic acid is isolated from a given sample using an acid guanidinium-phenol-chloroform extraction method and poly(A)<sup>+</sup> mRNA is isolated by oligo-dT column chromatography or by using (dT)<sub>n</sub> magnetic beads (*see, e.g.*, Sambrook et al., and Ausubel et al., *supra*).

In alternative embodiments, it is not necessary to isolate nucleic acids (*e.g.*, total or polyA<sup>+</sup> RNA) from the biological sample prior to carrying out amplification, hybridization or other assays. These embodiments have certain advantages when hTERT RNA is to be measured, because they reduce the possibility of loss of hTERT mRNA during isolation and handling. For example, many amplification techniques such as PCR and RT-PCR (reverse-transcriptase PCR) can be carried out using permeabilized cells (histological specimens and FACS analyses), whole lysed



cells, or crude cell fractions such as certain cell extracts. Preferably, steps are taken to preserve the integrity of the target nucleic acid (e.g., mRNA) if necessary (e.g., addition of RNAase inhibitors). Amplification and hybridization assays can also be carried out *in situ*, for example, in thin tissue sections from a biopsy sample or from a cell  
5 monolayer (e.g., blood cells or disaggregated tissue culture cells). Amplification can also be carried out in an intact whole cell or fixed cells. For example, PCR, RT-PCR, or LCR amplification methods may be carrier out, as is well known in the art, *in situ*, e.g., using a polymerase or ligase, a primer or primer(s), and (deoxy)ribonucleoside triphosphates (if a polymerase is employed), and reverse transcriptase and primer (if  
10 RNA to be transcribed and the cDNA to be is detected) on fixed, permeabilized, or microinjected cells to amplify target hTERT RNA or DNA. Cells containing hTERT RNA (e.g., telomerase positive cells) or an hTERT DNA sequence of interest can then be detected. This method is often useful when fluorescently-labeled dNTPs, primers, or other components are used in conjunction with microscopy, FACS analysis or the  
15 equivalent.

## 2) AMPLIFICATION BASED ASSAYS

In one embodiment, the assays of the present invention are amplification-based assays for detection of a hTERT gene or gene product. In an  
20 amplification based assay, all or part of an hTERT gene or transcript (e.g., mRNA or cDNA; hereinafter also referred to as "target") is amplified, and the amplification product is then detected directly or indirectly. When there is no underlying gene or gene product to act as a template, no amplification product is produced, or amplification is non-specific and typically there is no single amplification product. In  
25 contrast, when the underlying gene or gene product is present, the target sequence is amplified, providing an indication of the presence and/or quantity of the underlying gene or mRNA. Amplification-based assays are well known to those of skill in the art.

The present invention provides a wide variety of primers and probes for detecting hTERT genes and gene products. Such primers and probes are sufficiently  
30 complementary to the hTERT gene or gene product to hybridize to the target nucleic

acid. Primers are typically at least 6 bases in length, usually between about 10 and about 100 bases, typically between about 12 and about 50 bases, and often between about 14 and about 25 bases in length. One of skill, having reviewed the present disclosure, will be able, using routine methods, to select primers to amplify all, or any portion, of the hTRT gene or gene product, or to distinguish between variant gene products, hTRT alleles, and the like. **Table 2** lists illustrative primers useful for PCR amplification of the hTRT, or specific hTRT gene products or regions. As is known in the art, single oligomers (e.g., U.S. Pat. No. 5,545,522), nested sets of oligomers, or even a degenerate pool of oligomers may be employed for amplification, e.g., as illustrated by the amplification of the *Tetrahymena* TRT cDNA as described in the above-cited priority documents.

The invention provides a variety of methods for amplifying and detecting an hTRT gene or gene product, including the polymerase chain reaction (including all variants, e.g., reverse-transcriptase-PCR; the Sunrise Amplification System (Oncor, Inc, Gaithersburg MD) and numerous others known in the art). In one illustrative embodiment, PCR amplification is carried out in a solution containing the nucleic acid sample (e.g., cDNA obtained through reverse transcription of hTRT RNA), dATP, dCTP, dGTP and dTTP (i.e., Pharmacia LKB Biotechnology, NJ), the hTRT-specific PCR primer(s), 1 unit/ Taq polymerase (Perkin Elmer, Norwalk CT), 100  $\mu$ M dNTPs, 1x PCR buffer (50 mM KCl, 10 mM Tris, pH 8.3 at room temperature, 1.5 mM  $MgCl_2$ , 0.01% gelatin) with the amplification run for about 30 cycles at 94° for 45 sec, 55° for 45 sec and 72° for 90 sec at 95° for 1 minute, followed by about 30 cycles at 94° for 45 sec, 55° for 45 sec and 72° for 90 sec. However, as will be appreciated, numerous variations may be made to optimize the PCR amplification for any particular reaction.

Other suitable target amplification methods include the ligase chain reaction (LCR) (e.g., Wu and Wallace, 1989, *Genomics* 4:560; Landegren *et al.*, 1988, *Science*, 241: 1077, Barany, 1991, *Proc. Natl. Acad. Sci. USA* 88:189 and Barringer *et al.*, 1990, *Gene*, 89: 117); strand displacement amplification (SDA) (e.g., Walker *et al.*, 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89:392-396); transcription amplification (e.g.,

Kwoh *et al.*, 1989, *Proc. Natl. Acad. Sci. USA*, 86: 1173); self-sustained sequence replication (3SR) (e.g., Fahy *et al.*, 1992, *PCR Methods Appl.* 1:25, Guatelli *et al.*, 1990, *Proc. Nat. Acad. Sci. USA*, 87: 1874); the nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario; e.g., Compton, 1991, *Nature* 350:91); the transcription-based amplification system (TAS); and the self-sustained sequence replication system (SSR). Each of the aforementioned publications is incorporated herein by reference. One useful variant of PCR is PCR ELISA (e.g., Boehringer Mannheim Cat. No. 1 636 111) in which digoxigenin-dUTP is incorporated into the PCR product. The PCR reaction mixture is denatured and hybridized with a biotin-labeled oligonucleotide designed to anneal to an internal sequence of the PCR product. The hybridization products are immobilized on strepavidin coated plates and detected using anti-digoxigenin antibodies. Examples of techniques sufficient to direct persons of skill through *in vitro* amplification methods are found PCR TECHNOLOGY: PRINCIPLES AND APPLICATIONS FOR DNA AMPLIFICATION, H. Erlich, Ed. Freeman Press, New York, NY (1992); PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, eds. Innis, Gelfand, Snisky, and White, Academic Press, San Diego, CA (1990); Mattila *et al.*, 1991, *Nucleic Acids Res.* 19: 4967; Eckert and Kunkel, (1991) PCR METHODS AND APPLICATIONS 1: 17; PCR, eds. McPherson, Quirk, and Taylor, IRL Press, Oxford; U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188; Barringer *et al.*, 1990, *Gene*, 89:117; Kwoh *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86:1173; Guatelli *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87:1874; Lomell *et al.*, 1989, *J. Clin. Chem.*, 35:1826, each of which is incorporated herein for all purposes.

Amplified products may be directly analyzed (e.g., by size as determined by gel electrophoresis); by hybridization to a target nucleic acid immobilized on a solid support such as a bead, membrane, slide, or chip; by sequencing; immunologically (e.g., by PCR-ELISA), by detection of a fluorescent, phosphorescent, or radioactive signal, or any of a variety of other well-known means. For example, an illustrative example of a detection method uses PCR primers augmented with hairpin loops linked to fluorescein and a benzoic acid derivative that serves as a quencher, such that fluorescence is emitted only when the primers unfold to bind their targets and

replication occurs.

Because hTRT mRNA is typically expressed as an extremely rare transcript, present at very low levels even in telomerase positive cells, it is often desirable to optimize or increase the signal resulting from the amplification step. One way to do this is to increase the number of cycles of amplification. For example, although 20-25 cycles are adequate for amplification of most mRNAs using the polymerase chain reaction, detection of hTRT mRNA in many samples can require as many as 30 to 35 cycles of amplification, depending on detection format. It will be recognized that judicious choice of the amplification conditions including the number of amplification cycles can be used to design an assay that results in an amplification product only when there is a threshold amount of template in the test sample (i.e., so that only samples with a high level of hTRT mRNA give a "positive" result). In addition, methods are known to increase signal produced by amplification of the target sequence. Methods for augmenting the ability to detect the amplified target include signal amplification system such as: branched DNA signal amplification (e.g., U.S. Pat. No. 5,124,246; Urdea, 1994, *Bio/Tech.* 12:926); tyramide signal amplification (TSA) system (Du Pont); catalytic signal amplification (CSA) (Dako); Q Beta Replicase systems (Tyagi et al., 1996, *Proc. Nat. Acad. Sci. USA*, 93: 5395 ), or the like.

One of skill in the art will appreciate that whatever amplification method is used, a variety of quantitative methods known in the art may be used if quantitation is desired. For example, when desired, two or more polynucleotides may be co-amplified in a single sample. This method may be used as a convenient method of quantitating the amount of hTRT mRNA in a sample, because the reverse transcription and amplification reactions are carried out in the same reaction for a test and control polynucleotide. The co-amplification of the control polynucleotide (usually present at a known concentration or copy number) can be used for normalization to the cell number in the sample as compared to the amount of hTRT in the sample. Suitable control polynucleotides for co-amplification reactions include DNA, RNA expressed from housekeeping genes, constitutively expressed genes, and *in vitro* synthesized RNAs or DNAs added to the reaction mixture. Endogenous control polynucleotides are those

that are already present in the sample, while exogenous control polynucleotides are added to a sample, creating a "spiked" reaction. Illustrative control RNAs include  $\beta$ -actin RNA, GAPDH RNA, snRNAs, hTR, and endogenously expressed 28S rRNA (see Khan *et al.*, 1992, *Neurosci. Lett.* 147:114). Exogenous control polynucleotides include a synthetic AW106 cRNA, which may be synthesized as a sense strand from pAW106 by T7 polymerase. It will be appreciated that for the co-amplification method to be useful for quantitation, the control and test polynucleotides must typically both be amplified in a linear range. Detailed protocols for quantitative PCR may be found in PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, Innis *et al.*, Academic Press, Inc. N.Y., (1990) and Ausubel *et al.*, *supra* (Unit 15) and Diaco, R. (1995) *Practical Considerations for the Design of Quantitative PCR Assays*, in PCR STRATEGIES, pg. 84-108, Innis *et al.* eds, Academic Press, New York.

Depending on the sequence of the endogenous or exogenous standard, different primer sets may be used for the co-amplification reaction. In one method, called competitive amplification, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers used for amplification of the target nucleic acid (one pair of 2 primers). In an alternative embodiment, known as non-competitive competition, the control sequence and the target sequence (e.g., hTERT cDNA) are amplified using different primers (i.e., 2 pairs of 2 primers). In another alternative embodiment, called semi-competitive amplification, three primers are used, one of which is hTERT-specific, one of which is control specific, and one of which is capable of annealing to both the target and control sequences. Semi-competitive amplification is described in U.S. Patent No. 5,629,154, which is incorporated herein by reference.

### 3) HYBRIDIZATION-BASED ASSAYS

#### a) GENERALLY

A variety of methods for specific DNA and RNA measurement using nucleic acid hybridization techniques are known to those of skill in the art (see Sambrook *et al.*, *supra*). Hybridization based assays refer to assays in which a probe

nucleic acid is hybridized to a target nucleic acid. Usually the nucleic acid hybridization probes of the invention are entirely or substantially identical to a contiguous sequence of the hTRT gene or RNA sequence. Preferably nucleic acid probes are at least about 10 bases, often at least about 20 bases, and sometimes at least about 200 bases or more. Methods of selecting nucleic acid probe sequences for use in nucleic acid hybridization are discussed in Sambrook et al., *supra*. In some formats, at least one of the target and probe is immobilized. The immobilized nucleic acid may be DNA, RNA, or another oligo- or poly-nucleotide, and may comprise natural or non-naturally occurring nucleotides, nucleotide analogs, or backbones. Such assays may be in any of several formats including: Southern, Northern, dot and slot blots, high-density polynucleotide or oligonucleotide arrays (e.g., GeneChips™ Affymetrix), dip sticks, pins, chips, or beads. All of these techniques are well known in the art and are the basis of many commercially available diagnostic kits. Hybridization techniques are generally described in Hames et al., ed., NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH IRL Press, (1985); Gall and Pardue *Proc. Natl. Acad. Sci., U.S.A.*, 63: 378-383 (1969); and John et al., *Nature*, 223: 582-587 (1969).

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, one common format is direct hybridization, in which a target nucleic acid is hybridized to a labeled, complementary probe. Typically, labeled nucleic acids are used for hybridization, with the label providing the detectable signal. One method for evaluating the presence, absence, or quantity of hTRT mRNA is carrying out a Northern transfer of RNA from a sample and hybridization of a labeled hTRT specific nucleic acid probe, as illustrated in **Example 2**. As was noted *supra*, hTRT mRNA, when present at all, is present in very low quantities in most cells. Therefore, when Northern hybridization is used, it will often be desirable to use an amplification step (or, alternatively, large amounts of starting RNA). A useful method for evaluating the presence, absence, or quantity of DNA encoding hTRT proteins in a sample involves a Southern transfer and sample and hybridization of a labeled hTRT specific nucleic acid probe.

Other common hybridization formats include sandwich assays and

competition or displacement assays. Sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid in solution. The biological or clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

#### b) CHIP-BASED AND SLIDE-BASED ASSAYS

The present invention also provides probe-based hybridization assays for hTERT gene products employing arrays of immobilized oligonucleotide or polynucleotides to which an hTERT nucleic acid can hybridize (i.e., to some, but usually not all or even most, of the immobilized oligo- or poly-nucleotides). High density oligonucleotide arrays or polynucleotide arrays provide a means for efficiently detecting the presence and characteristics (e.g., sequence) of a target nucleic acid (e.g., hTERT gene, mRNA, or cDNA). Techniques are known for producing arrays containing thousands of oligonucleotides complementary to defined sequences, at defined locations on a surface using photolithographic techniques for synthesis *in situ* (see, e.g., U.S. Patent Nos. 5,578,832; 5,556,752; and 5,510,270; Fodor et al., 1991, *Science* 251:767; Pease et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:5022; and Lockhart et al., 1996, *Nature Biotech* 14:1675) or other methods for rapid synthesis and deposition of defined oligonucleotides (Blanchard et al., 1996, *Biosensors & Bioelectronics* 11:687). When these methods are used, oligonucleotides (e.g., 20-mers) of known sequence are synthesized directly on a surface such as a derivatized glass slide. Usually, the array produced is redundant, having several oligonucleotide probes on the chip specific for the hTERT polynucleotide to be detected.

Combinations of oligonucleotide probes can be designed to detect alternatively spliced mRNAs, or to identify which of various hTERT alleles is expressed in a particular sample.

In one illustrative embodiment, cDNA prepared by reverse transcription

of total RNA from a test cell is amplified (e.g., using PCR). Typically the amplification product is labeled, e.g., by incorporation of a fluorescently labeled dNTP. The labeled cDNAs are then hybridized to a chip comprising oligonucleotide probes complementary to various subsequences of the hTRT gene. The positions of hybridization are determined (e.g., in accordance with the general methods of Shalon et al., 1996, *Genome Research* 6:639 or Schena et al., 1996, *Genome Res.* 6:639), and sequence (or other information) deduced from the hybridization pattern, by means well known in the art.

In one embodiment, two cDNA samples, each labeled with a different fluorescent group, are hybridized to the same chip. The ratio of the hybridization of each labeled sample to sites complementary to the hTRT gene are then assayed. If both samples contain the same amount of hTRT mRNA, the ratio of the two fluors will be 1:1 (it will be appreciated that the signal from the fluors may need to be adjusted to account for any difference in the major sensitivity of the fluors). In contrast, if one sample is from a healthy (or control) tissue and the second sample is from a cancerous tissue the fluor used in the second sample will predominate.

### c) IN SITU HYBRIDIZATION

An alternative means for detecting expression of a gene encoding an hTRT protein is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer et al., *METHODS ENZYMOL.*, 152: 649-660 (1987) and Ausubel et al., *supra*. In an *in situ* hybridization assay, cells or tissue specimens are fixed to a solid support, typically in a permeabilized state, typically on a glass slide. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled nucleic acid probes (e.g., <sup>35</sup>S-labeled riboprobes, fluorescently labeled probes) completely or substantially complementary to hTRT. Free probe is removed by washing and/or nuclease digestion, and bound probe is visualized directly on the slide by autoradiography or an appropriate imaging techniques, as is known in the art.



#### 4) SPECIFIC DETECTION OF VARIANTS

As noted *supra* and illustrated in the Examples (e.g., **Example 9**), amplification primers or probes can be selected to provide amplification products that span specific deletions, truncations, and insertions, thereby facilitating the detection of specific variants or abnormalities in the hTERT mRNA.

One example of an hTERT variant gene product that may be detected is an hTERT RNA such as a product (SEQ. ID. NO: 4) described *supra* and in **Example 9**. The biological function, if any, of the  $\Delta 182$  variant(s) is not known; however, the truncated hTERT protein putatively encoded by the variant may be involved in regulation of telomerase activity, e.g., by assembling a non-functional telomerase RNP that titrates telomerase components. Alternatively, negative regulation of telomerase activity could be accomplished by directing hTERT pre-mRNA (nascent mRNA) processing in a manner leading to elimination of the mRNA and reducing hTERT mRNA levels. For these and other reasons, the ability to detect  $\Delta 182$  variants is useful. In addition, it will sometimes be desirable, in samples in which two species of hTERT RNA are present (such as a  $\Delta 182$  hTERT RNA and hTERT encoding the full-length hTERT protein) to compare their relative and/or absolute abundance.

The invention provides a variety of methods for detection of  $\Delta 182$  variants. For example, amplification using primer pairs spanning the 182 basepair deletion will result in different sized products corresponding to the deleted and undeleted hTERT RNAs, if both are present, which can be distinguished on the basis of size (e.g., by gel electrophoresis). Examples of primer pairs useful for amplifying the region spanning the 182 bp deletion include TCP1.14 and TCP1.15 (primer set 1), or TCP1.25 and bTCP6 (primer set 2) (see **Table 2**). These primer pairs can be used individually or in a nested PCR experiment where primer set 1 is used first. It will also be apparent to one of skill that hybridization methods (e.g., Northern hybridization) or RNase protection assays using an hTERT nucleic acid probe of the invention can be used to detect and distinguish hTERT RNA variants.

Another suitable method entails PCR amplification (or the equivalent)

using three primers. Analogous to the semi-competitive quantitative PCR method described in greater detail *supra*, one primer is specific to each of the hTRT RNA species (e.g., as illustrated in **Table 4**) and one primer is complementary to both species (e.g., TCP1.25 (2270-2288)). An example of a primer specific to **SEQ. ID. NO.: 1** is one that anneals within the 182 nucleotide sequence (i.e., nucleotides 2345 to 2526 of **SEQ. ID. NO: 1**), e.g., TCP1.73 (2465-2445). For example, a primer specific to **SEQ. ID. No. 4** (a  $\Delta 182$  variant) is one that anneals at nucleotides 2358 to 2339 of **SEQ. ID. NO: 4** (i.e., the site corresponding to the 182 nucleotide insertion in **SEQ. ID. NO: 1**). The absolute abundance of the  $\Delta 182$  hTRT mRNA species or its relative abundance compared to the species encoding the full-length hTRT protein can be analyzed for correlation to cell state (e.g., capacity for indefinite proliferation). It will be appreciated that numerous other primers may be selected based on the present disclosure.

**TABLE 4****ILLUSTRATIVE PRIMERS**

$\Delta 182$  species (e.g., **SEQ. ID. NO. 4**) specific primer:

5'-GGCACTGGACGTAGGACGTG-3

hTRT (**SEQ. ID. NO. 1**) specific primer (TCP1.73):

5'-CACTGCTGGCCTCATTTCAGGG-3

Common (forward) primer (TCP1.25):

5'-TACTGCGTGCGTCGGTATG-3'

Other variant hTRT genes or gene products that may be detected include those characterized by premature stop codons, deletions, substitutions or insertions.

Deletions can be detected by the decreased size of the gene, mRNA transcript, or cDNA. Similarly, insertions can be detected by the increased size of the gene, mRNA transcript, of cDNA. Insertions and deletions could also cause shifts in the reading frame that lead to premature stop codons or longer open reading frames. Substitutions can be detected by probe hybridization. These alterations are detected by observing changes in the size of the variant hTRT polypeptide or by hybridization or specific

amplification as appropriate. Alternatively, mutations can be determined by sequencing of the gene or gene product according to standard methods. In addition, and as noted above, amplification assays and hybridization probes can be selected to target particular abnormalities specifically. For example, where the variation is a deletion, nucleic acid probes or amplification primers can be selected that specifically hybridize to or amplify, respectively, the region encompassing the deletion, substitution, or insertion. Where the hTERT gene harbors such a mutation, the probe will either (1) fail to hybridize or the amplification reaction will fail to provide specific amplification or cause a change in the size of the amplification product or hybridization signal; or (2) the probe or amplification reaction encompasses the entire deletion or either end of the deletion (deletion junction); or (3) similarly, probes and amplification primers can be selected that specifically target point mutations or insertions.

#### Detection of mutant hTERT alleles

Mutations in the hTERT gene could be responsible for disease initiation or could contribute to a disease condition. Alterations of the genomic DNA of hTERT could affect levels of gene transcription, change amino acid residues in the hTERT protein, cause truncated hTERT polypeptides to be produced, alter pre-mRNA processing pathways (which can alter hTERT mRNA levels), and cause other consequences as well.

Alterations of genomic DNA in non-hTERT loci can also affect expression of hTERT or telomerase by altering the enzymes or cellular processes that are responsible for regulating hTERT, hTR, and telomerase-associated protein expression and processing and RNP assembly and transport. Alterations which affect hTERT expression, processing, or RNP assembly could be important for cancer progression, for diseases of aging, for DNA damage diseases, and others.

Detection of mutations in hTERT mRNA or its gene and gene control elements can be accomplished in accordance with the methods herein in multiple ways. Illustrative examples include the following: A technique termed primer screening can be employed; PCR primers are designed whose 3' termini anneal to nucleotides in a sample DNA (or RNA) that are possibly mutated. If the DNA (or RNA) is amplified

by the primers then the 3' termini matched the nucleotides in the gene; if the DNA is not amplified, then one or both termini did not match the nucleotides in the gene, indicating a mutation was present. Restriction fragment length polymorphism, RFLP (Pourzand, C., Cerutti, P. (1993) Mutat. Res 288: 113-121), is another technique that can be applied in the present method. A Southern blot of human genomic DNA digested with various restriction fragments is probed with an hTERT specific probe, differences in the fragment sizes between the sample and a control indicate an alteration of the experimental sample, usually an insertion or deletion. Single strand conformation polymorphism, SSCP (Orrita, M., et al. (1989) PNAS USA 86:2766-70), is another technique that can be applied in the present method. SSCP is based on the differential migration of denatured wild-type and mutant single-stranded DNA (usually generated by PCR). Single-stranded DNA will take on a three-dimensional conformation that is sequence-specific. Sequence differences as small as a single base change can result in a mobility shift on a nondenaturing gel. SSCP is one of the most widely used mutation screening methods because of its simplicity. Denaturing Gradient Gel Electrophoresis, DGGE (Myers, R. M., Maniatis, T. and Lerman, L., (1987) Methods in Enzymology, 155: 501-527), is another technique that can be applied in the present method. DGGE identifies mutations based on the melting behavior of double-stranded DNA. Specialized denaturing electrophoresis equipment is utilized to observe the melting profile of experimental and control DNAs, a DNA containing a mutation will have a different mobility compared to the control in these gel systems. Many other techniques exist which are known by those skilled in the art, the examples discussed illustrate commonly employed methodology.

## 25                    5) KARYOTYPE ANALYSIS

The present invention further provides methods and reagents for karyotype or other chromosomal analysis using hTERT-sequence probes and/or detecting or locating hTERT gene sequences in chromosomes from a human patient, human cell line, or non-human cell. In one embodiment, amplification (i.e., change in copy number), deletion (i.e., partial deletion), insertion, substitution, or changes in the

chromosomal location (e.g., translocation) of an hTRT gene may be correlated with the presence of a pathological condition or a predisposition to developing a pathological condition (e.g., cancer).

5 It has been determined by the present inventors that, in normal human cells, the hTRT gene maps close to the telomere of chromosome 5p (see **Example 5**, *infra*). The closest STS marker was D5S678. The location can be used to identify markers that are closely linked to the hTRT gene. The markers can be used to identify YACs, STSs, cosmids, BACs, lambda or P1 phage, or other clones which contain hTRT genomic sequences or control elements. The markers or the gene location can be  
10 used to scan human tissue samples for alterations in the normal hTRT gene location, organization or sequence that is associated with the occurrence of a type of cancer or disease. This information can be used in a diagnostic or prognostic manner for the disease or cancer involved. Moreover, the nature of any alterations to the hTRT gene can be informative to the nature by which cells become immortal. For instance, a  
15 translocation event could indicate that activation of hTRT expression occurs in some cases by replacing the hTRT promoter with another promoter which directs hTRT transcription in an inappropriate manner. Methods and reagents of the invention of this type can be used to develop strategies to combat hTRT activation processes. The location may also be useful for determining the nature of hTRT gene repression in  
20 normal somatic cells, for instance, whether the location part of non-expressing heterochromatin. Nuclease hypersensitivity assays for distinguishing heterochromatin and euchromatin are described, for example, in Wu et al., 1979, *Cell* 16:797; Groudine and Weintraub, 1982, *Cell* 30:131 Gross and Garrard, 1988, *Ann. Rev. Biochem.* 57:159.

25 In one embodiment, alterations to the hTRT gene are identified by karyotype analysis, using any of a variety of methods known in the art. One useful technique is *in situ* hybridization (ISH). Typically, when *in situ* hybridization techniques are used for karyotype analysis, a detectable or detectably-labeled probe is hybridized to a chromosomal sample *in situ* to locate a hTRT gene sequence.  
30 Generally, ISH comprises one or more of the following steps: (1) fixation of the tissue,

cell or other biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target DNA (e.g., denaturation with heat or alkali), and to reduce nonspecific binding (e.g., by blocking the hybridization capacity of repetitive sequences, e.g., using human genomic DNA); (3) hybridization of one or more nucleic acid probes (e.g., conventional nucleic acids, PNAs, or other nucleic acid analogs) to the nucleic acid in the biological structure or tissue; (4) posthybridization washes to remove nucleic acid fragments not bound in the hybridization; and, (5) detection of the hybridized nucleic acid fragments. The reagent used in each of these steps and their conditions for use vary depending on the particular application. It will be appreciated that these steps can be modified in a variety of ways well known to those of skill in the art.

In one embodiment of ISH, the hTERT probe is labeled with a fluorescent label (fluorescent *in situ* hybridization; "FISH"). Typically, it is desirable to use dual color fluorescent *in situ* hybridization, in which two probes are utilized, each labeled by a different fluorescent dye. A test probe that hybridizes to the hTERT sequence of interest is labeled with one dye, and a control probe that hybridizes to a different region is labeled with a second dye. A nucleic acid that hybridizes to a stable portion of the chromosome of interest, such as the centromere region, can be used as the control probe. In this way, one can account for differences between efficiency of hybridization from sample to sample.

The ISH methods for detecting chromosomal abnormalities (e.g., FISH) can be performed on nanogram quantities of the subject nucleic acids. Paraffin embedded normal tissue or tumor sections can be used, as can fresh or frozen material, tissues, or sections. Because FISH can be applied to the limited material, touch preparations prepared from uncultured primary tumors can also be used (*see, e.g., Kallioniemi et al., 1992, Cytogenet. Cell Genet.* 60:190). For instance, small biopsy tissue samples from tumors can be used for touch preparations (*see, e.g., Kallioniemi et al., supra*). Small numbers of cells obtained from aspiration biopsy or cells in bodily fluids (e.g., blood, urine, sputum and the like) can also be analyzed. For prenatal diagnosis, appropriate samples will include amniotic fluid, maternal blood, and the like.

Useful hybridization protocols applicable to the methods and reagents disclosed here are described in Pinkel et al., 1988, *Proc. Natl. Acad. Sci. USA*, 85:9138; EPO Pub. No. 430,402; Choo, ed., *METHODS IN MOLECULAR BIOLOGY VOL. 33: IN SITU HYBRIDIZATION PROTOCOLS*, Humana Press, Totowa, New Jersey, (1994); and  
5 Kallioniemi et al., *supra*.

Other techniques useful for karyotype analysis include, for example, techniques such as quantitative Southern blotting, quantitative PCR, or comparative genomic hybridization (Kallioniemi et al., 1992, *Science*, 258:818), using the hTRT probes and primers of the invention which may be used to identify amplification,  
10 deletion, insertion, substitution or other rearrangement of hTRT sequences in chromosomes in a biological sample.

## G. TRT POLYPEPTIDE ASSAYS

### 1) GENERALLY

15 The present invention provides methods and reagents for detecting and quantitating hTRT polypeptides. These methods include analytical biochemical methods such as electrophoresis, mass spectroscopy, gel shift, capillary electrophoresis, chromatographic methods such as size exclusion chromatography, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion  
20 chromatography, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, mass spectrometry, and others described below and apparent to those of skill in the art upon review of this disclosure.

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### 2) ELECTROPHORETIC ASSAYS

In one embodiment, the hTRT polypeptides are detected in an electrophoretic protein separation; in one aspect, a two-dimensional electrophoresis system is employed. Means of detecting proteins using electrophoretic techniques are  
30 well known to those of skill in the art (*see generally*, R. Scopes (1982) *PROTEIN*

PURIFICATION, Springer-Verlag, N.Y.; Deutscher, (1990) METHODS IN ENZYMOLOGY VOL. 182: GUIDE TO PROTEIN PURIFICATION, Academic Press, Inc., N.Y.).

In a related embodiment, a mobility shift assay (see, e.g., Ausubel et al., *supra*) is used. For example, labeled-hTR will associate with hTRT and migrate with altered mobility upon electrophoresis in a nondenaturing polyacrylamide gel or the like. Thus, for example, if a labeled hTR probe is mixed with a sample containing hTRT, or coexpressed with hTRT (e.g., in a cell-free expression system) the presence of hTRT protein (or a polynucleotide encoding hTRT) in the sample will result in a detectable alteration of hTR mobility.

### 3) IMMUNOASSAYS

#### a) GENERALLY

The present invention also provides methods for detection of hTRT polypeptides employing one or more antibody reagents of the invention (i.e., immunoassays). As used herein, an immunoassay is an assay that utilizes an antibody (as broadly defined herein and specifically includes fragments, chimeras and other binding agents) that specifically binds an hTRT polypeptide or epitope. Antibodies of the invention may be made by a variety of means well known to those of skill in the art, e.g., as described *supra*.

A number of well established immunological binding assay formats suitable for the practice of the invention are known (see, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). See, e.g., METHODS IN CELL BIOLOGY VOLUME 37: ANTIBODIES IN CELL BIOLOGY, Asai, ed. Academic Press, Inc. New York (1993); BASIC AND CLINICAL IMMUNOLOGY 7th Edition, Stites & Terr, eds. (1991); Harlow and Lane, *supra* [e.g., Chapter 14], and Ausubel et al., *supra*, [e.g., Chapter 11], each of which is incorporated by reference in its entirety and for all purposes. Typically, immunological binding assays (or immunoassays) utilize a "capture agent" to specifically bind to and, often, immobilize the analyte. In one embodiment, the capture agent is a moiety that specifically binds to an hTRT polypeptide or subsequence, such as an anti-hTRT antibody. In an alternative embodiment, the capture agent may bind



an hTRT-associated protein or RNA under conditions in which the hTRT-associated molecule remains bound to the hTRT (such that if the hTRT-associated molecule is immobilized the hTRT protein is similarly immobilized). It will be understood that in assays in which an hTRT-associated molecule is captured the associated hTRT protein will usually be detected, e.g., using an anti-hTRT antibody or the like. Immunoassays for detecting protein complexes are known in the art (see, e.g., Harlow and Lane, *supra*, at page 583).

Usually the hTRT gene product being assayed is detected directly or indirectly using a detectable label. The particular label or detectable group used in the assay is usually not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody or antibodies used in the assay. The label may be covalently attached to the capture agent (e.g., an anti-TRT antibody), or may be attached to a third moiety, such as another antibody, that specifically binds to, e.g., : the hTRT polypeptide (at a different epitope than recognized by the capture agent), the capture agent (e.g., an anti-(first antibody) immunoglobulin); an anti-TRT antibody; an antibody that binds an anti-TRT antibody; or, an antibody/telomerase complex (e.g., via binding to an associated molecule such as a telomerase-associated protein). Other proteins capable of binding an antibody used in the assay, such as protein A or protein G, may also be labeled. In some embodiments, it will be useful to use more than one labeled molecule (i.e., ones that can be distinguished from one another). In addition, when the target bound (e.g., immobilized) by the capture agent (e.g., anti-hTRT antibody) is a complex (i.e., a complex of hTRT and a TRT-associated protein, hTR, or other TRT associated molecule), a labeled antibody that recognizes the protein or RNA associated with the hTRT protein may be used. When the complex is a protein-nucleic acid complex (e.g., TRT-hTR), the reporter molecule may be a polynucleotide or other molecule (e.g., enzyme) that recognizes the RNA component of the complex.

Some immunoassay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples

comprising the target antibodies. In this format, the components do not need to be labeled, and the presence of the target antibody can be detected by simple visual inspection.

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#### **b) NON-COMPETITIVE ASSAY FORMATS**

The present invention provides methods and reagents for competitive and noncompetitive immunoassays for detecting hTRT polypeptides. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case hTRT) is directly measured. One such assay is a two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the hTRT protein. See, e.g., Maddox et al., 1983, *J. Exp. Med.*, 158:1211 for background information. In one preferred "sandwich" assay, the capture agent (e.g., an anti-TRT antibody) is bound directly to a solid substrate where it is immobilized. These immobilized antibodies then capture any hTRT protein present in the test sample. The hTRT thus immobilized can then be labeled, i.e., by binding to a second anti-hTRT antibody bearing a label. Alternatively, the second anti-hTRT antibody may lack a label, but be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second antibody alternatively can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

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#### **c) COMPETITIVE ASSAY FORMATS**

In competitive assays, the amount of hTRT protein present in the sample is measured indirectly by measuring the amount of an added (exogenous) hTRT displaced (or competed away) from a capture agent (e.g., anti-TRT antibody) by the hTRT protein present in the sample. In one competitive assay, a known amount of labeled hTRT protein is added to the sample and the sample is then contacted with a capture agent (e.g., an antibody that specifically binds hTRT protein). The amount of exogenous (labeled) hTRT protein bound to the antibody is inversely proportional to the concentration of hTRT protein present in the sample. In one embodiment, the

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antibody is immobilized on a solid substrate. The amount of hTRT protein bound to the antibody may be determined either by measuring the amount of hTRT protein present in an TRT/antibody complex, or alternatively by measuring the amount of remaining uncomplexed TRT protein. The amount of hTRT protein may be detected by providing a labeled hTRT molecule.

A hapten inhibition assay is another example of a competitive assay. In this assay hTRT protein is immobilized on a solid substrate. A known amount of anti-TRT antibody is added to the sample, and the sample is then contacted with the immobilized hTRT protein. In this case, the amount of anti-TRT antibody bound to the immobilized hTRT protein is inversely proportional to the amount of hTRT protein present in the sample. The amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. In this aspect, detection may be direct, where the antibody is labeled, or indirect where the label is bound to a molecule that specifically binds to the antibody as described above.

#### **d) OTHER ASSAY FORMATS**

The invention also provides reagents and methods for detecting and quantifying the presence of hTRT in the sample by using an immunoblot (Western blot) format. In this format, hTRT polypeptides in a sample are separated from other sample components by gel electrophoresis (e.g., on the basis of molecular weight), the separated proteins are transferred to a suitable solid support (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and the support is incubated with anti-TRT antibodies of the invention. The anti-TRT antibodies specifically bind to hTRT or other TRT on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled sheep anti-mouse antibodies) or other labeling reagents that specifically bind to the anti-TRT antibody.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (e.g., antibodies) and release

encapsulated reagents or markers. The released chemicals can then be detected according to standard techniques (*see*, Monroe et al., 1986, *Amer. Clin. Prod. Rev.* 5:34).

As noted *supra*, assay formats using FACS (and equivalent instruments or methods) have advantages when measuring hTERT gene products in a heterogeneous sample (such as a biopsy sample containing both normal and malignant cells).

#### e) SUBSTRATES, SOLID SUPPORTS, MEMBRANES, FILTERS

As noted *supra*, depending upon the assay, various components, including the antigen, target antibody, or anti-hTERT antibody, may be bound to a solid surface or support (i.e., a substrate, membrane, or filter paper). Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.*, glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

5           If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of  
10       compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas (1970) *J. Biol. Chem.* 245 3059).

          In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific  
15       absorption of a compound to the surface.

          One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific  
20       binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk sometimes preferred. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For  
25       example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082.

## 30           **H) ASSAYS FOR ANTI-TRT ANTIBODIES**

The present invention also provides reagents and assays for detecting hTERT-specific immunoglobulins. In one embodiment, immobilized hTERT (e.g., recombinant hTERT bound to a microassay plate well) is incubated with serum from a patient under conditions in which anti-hTERT antibodies, if present, bind the immobilized hTERT. After washing to remove nonspecifically bound immunoglobulin, bound serum antibodies can be detected, if they are present, by adding detectably labeled anti-(human Ig) antibodies (alternative embodiments and variations are well known to those of skill in the art; see, e.g., Harlow, *supra*, at Ch. 14). These assays are useful for detecting anti-hTERT antibodies in any source including animal or human serum or a carrier such as saline. In one embodiment, the assays are used to detect or monitor an immune response to hTERT proteins in a patient, particularly an autoimmune (e.g., anti-telomerase) response. Anti-hTERT antibodies may be present in the serum or other tissues or fluids from a patient suffering from an autoimmune disease or other condition.

#### I) ASSAY COMBINATIONS

The diagnostic and prognostic assays described herein can be carried out in various combinations and can also be carried out in conjunction with other diagnostic or prognostic tests. For example, when the present methods are used to detect the presence of cancer cells in patient sample, the presence of hTERT can be used to determine the stage of the disease, whether a particular tumor is likely to invade adjoining tissue or metastasize to a distant location, and whether a recurrence of the cancer is likely. Tests that may provide additional information include microscopic analysis of biopsy samples, detection of antigens (e.g., cell-surface markers) associated with tumorigenicity (e.g., using histocytochemistry, FACS, or the like), imaging methods (e.g., upon administration to a patient of labeled anti-tumor antibodies), telomerase activity assays, telomere length assays, hTR assays, or the like. Such combination tests can provide useful information regarding the progression of a disease.

It will also be recognized that combinations of assays can provide useful

information. For example, and as noted above, assays for hTRT mRNA can be combined with assays for hTR (RNA) or TRAP assays to provide information about telomerase assembly and function.

5                   **J) KITS**

                  The present invention also provides kits useful for the screening, monitoring, diagnosis and prognosis of patients with a telomerase-related condition, or for determination of the level of expression of hTRT in cells or cell lines. The kits include one or more reagents for determining the presence or absence of a hTRT gene product (RNA or protein) or for quantifying expression of the hTRT gene. Preferred reagents include nucleic acid primers and probes that specifically bind to the hTRT gene, RNA, cDNA, or portions thereof, along with proteins, peptides, antibodies, and control primers, probes, oligonucleotides, proteins, peptides and antibodies. Other materials including enzymes (e.g., reverse transcriptases, DNA polymerases, ligases), buffers, reagents (labels, dNTPs), may be included.

                  The kits may include alternatively, or in combination with any of the other components described herein, an antibody that specifically binds to hTRT polypeptides or subsequences thereof. The antibody can be monoclonal or polyclonal. The antibody can be conjugated to another moiety such as a label and/or it can be immobilized on a solid support (substrate). The kit(s) may also contain a second antibody for detection of hTRT polypeptide/antibody complexes or for detection of hybridized nucleic acid probes, as well as one or more hTRT peptides or proteins for use as control or other reagents.

                  The antibody or hybridization probe may be free or immobilized on a solid support such as a test tube, a microtiter plate, a dipstick and the like. The kit may also contain instructional materials teaching the use of the antibody or hybridization probe in an assay for the detection of a predisposition for TRT. The kit may contain appropriate reagents for detection of labels, or for labelling positive and negative controls, washing solutions, dilution buffers and the like.

                  In one embodiment, the kit includes a primer pair for amplifying hTRT

mRNA. Such a kit may also include a probe for hTERT amplified DNA and/or a polymerase, buffer, dNTPs, and the like. In another, the kit comprises a probe, optionally a labeled probe. In another, the kit comprises an antibody.

## 5 X. GLOSSARY

The following terms are defined *infra* to provide additional guidance to one of skill in the practice of the invention: adjuvant, allele (& allelic sequence), amino acids (including hydrophobic, polar, charged), conservative substitution, control elements (& regulatory sequences), derivatized, detectable label, elevated level, epitope, favorable and unfavorable prognosis, fusion protein, gene product, hTERT, immortal, immunogen and immunogenic, nucleic acid (& polynucleotide), oligonucleotides (& oligomers), operably linked, polypeptide, probe (including nucleic acid probes & antibody probes), recombinant, selection system, sequence, specific binding, stringent hybridization conditions (& stringency), substantial identity (& substantial similarity), substantially pure (& substantially purified and isolated), telomerase-negative and telomerase-positive cells, telomerase catalytic activity, and telomerase-related.

As used herein, the term “**adjuvant**” refers to its ordinary meaning of any substance that enhances the immune response to an antigen with which it is mixed. Adjuvants useful in the present invention include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (Bacillus Calmette-Guerin) and *Corynebacterium parvum* are potentially useful adjuvants.

As used herein, the terms “**allele**” or “**allelic sequence**” refer to an alternative form of a nucleic acid sequence (i.e., a nucleic acid encoding hTERT protein). Alleles result from mutations (i.e., changes in the nucleic acid sequence), and generally produce altered and/or differently regulated mRNAs or polypeptides whose structure



and/or function may or may not be altered. Common mutational changes that give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides that may or may not affect the encoded amino acids. Each of these types of changes may occur alone, in combination with the others, or one or more times within a given gene, chromosome or other cellular nucleic acid. Any given gene may have no, one or many allelic forms. As used herein, the term "allele" refers to either or both a gene or an mRNA transcribed from the gene.

As used herein, "**amino acids**" are sometimes specified using the standard one letter code: Alanine (A), Serine (S), Threonine (T), Aspartic acid (D), Glutamic acid (E) Asparagine (N), Glutamine (Q), Arginine (R), Lysine (K), Isoleucine (I), Leucine (L), Methionine (M), Valine (V), Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Proline (P), Glycine (G), Histidine (H), Cysteine (C). Synthetic and non-naturally occurring amino acid analogues (and/or peptide linkages) are included.

As used herein, "**Hydrophobic amino acids**" refers to A, L, I, V, P, F, W, and M. As used herein, "**polar amino acids**" refers to G, S, T, Y, C, N, and Q. As used herein, "**charged amino acids**" refers to D, E, H, K, and R.

As used herein, "**conservative substitution**", when describing a protein refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, *etc.*) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N),

Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W) (see also, Creighton (1984) *Proteins*, W.H. Freeman and Company). One of skill in the art will appreciate that the above-identified substitutions are not the only possible conservative substitutions. For example, one may regard all charged amino acids as conservative substitutions for each other whether they are positive or negative. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations". One can make a "conservative substitution" in a recombinant protein by utilizing one or more codons that differ from the codons employed by the native or wild-type gene. In this instance, a conservative substitution also includes substituting a codon for an amino acid with a different codon for the same amino acid.

As used herein, "**control elements**" or "**regulatory sequences**" include enhancers, promoters, transcription terminators, origins of replication, chromosomal integration sequences, 5' and 3' untranslated regions, to which proteins or other biomolecules interact to carry out transcription and translation. For eukaryotic cells, the control sequences will include a promoter and preferably an enhancer, e.g., derived from immunoglobulin genes, SV40, cytomegalovirus, and a polyadenylation sequence, and may include splice donor and acceptor sequences. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used

As used herein, a "**derivatized**" polynucleotide, oligonucleotide, or nucleic acid," refers to an oligo- and polynucleotides that comprises a derivatized substituent. In some embodiments, the substituent is substantially non-interfering with respect to hybridization to complementary polynucleotides. Derivatized oligo- or polynucleotides that have been modified with appended chemical substituents (e.g., by modification of an already synthesized oligo- or poly-nucleotide, or by incorporation of

a modified base or backbone analog during synthesis) may be introduced into a metabolically active eukaryotic cell to hybridize with an hTRT DNA, RNA, or protein where they produce an alteration or chemical modification to a local DNA, RNA, or protein. Alternatively, the derivatized oligo or polynucleotides may interact with and alter hTRT polypeptides, telomerase-associated proteins, or other factors that interact with hTRT DNA or hTRT gene products, or alter or modulate expression or function of hTRT DNA, RNA or protein. Illustrative attached chemical substituents include: europium (III) texaphyrin, cross-linking agents, psoralen, metal chelates (e.g., iron/EDTA chelate for iron catalyzed cleavage), topoisomerases, endonucleases, exonucleases, ligases, phosphodiesterases, photodynamic porphyrins, chemotherapeutic drugs (e.g., adriamycin, doxorubicin), intercalating agents, base-modification agents, immunoglobulin chains, and oligonucleotides. Iron/EDTA chelates are chemical substituents often used where local cleavage of a polynucleotide sequence is desired (Hertzberg et al., 1982, *J. Am. Chem. Soc.* 104: 313; Hertzberg and Dervan, 1984, *Biochemistry* 23: 3934; Taylor et al., 1984, *Tetrahedron* 40: 457; Dervan, 1986, *Science* 232: 464. Illustrative attachment chemistries include: direct linkage, e.g., via an appended reactive amino group (Corey and Schultz (1988) *Science* 238: 1401, which is incorporated herein by reference) and other direct linkage chemistries, although streptavidin/biotin and digoxigenin/anti-digoxigenin antibody linkage methods may also be used. Methods for linking chemical substituents are provided in U.S. Patents 5,135,720, 5,093,245, and 5,055,556, which are incorporated herein by reference. Other linkage chemistries may be used at the discretion of the practitioner.

As used herein, a “**detectable label**” has the ordinary meaning in the art and refers to an atom (e.g., radionuclide), molecule (e.g., fluoroscein), or complex, that is or can be used to detect (e.g., due to a physical or chemical property), indicate the presence of a molecule or to enable binding of another molecule to which it is covalently bound or closely associated. The term “label” also refers to covalently bound or closely associated molecules (e.g., a biomolecule such as an enzyme) that act on a substrate to produce a detectable atom, molecule or complex. Detectable labels

suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Labels useful in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, enhanced green fluorescent protein, lissamine, phycoerythrin, Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX [Amersham], SyBR Green I & II [Molecular Probes], and the like), radiolabels (e.g.,  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$ ), enzymes (e.g., hydrolases, particularly phosphatases such as alkaline phosphatase, esterases and glycosidases, or oxidoreductases, particularly peroxidases such as horse radish peroxidase, and others commonly used in an ELISA), substrates, cofactors, inhibitors, chemiluminescent groups, chromogenic agents, and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, *etc.*) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels and chemiluminescent labels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light (e.g., as in fluorescence-activated cell sorting). Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either inherently detectable or covalently bound to a signal generating system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-

ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody. The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter, photographic film as in autoradiography, or storage phosphor imaging. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Also, simple colorimetric labels may be detected by observing the color associated with the label. It will be appreciated that when pairs of fluorophores are used in an assay, it is often preferred that they have distinct emission patterns (wavelengths) so that they can be easily distinguished.

The phrase “**elevated level**” refers to an amount of hTERT gene product (or other specified substance or activity) in a cell that is elevated or higher than the level in a reference standard, e.g., for diagnosis, the level in normal, telomerase-negative cells in an individual or in other individuals not suffering from the condition, and for prognosis, the level in tumor cells from a variety of grades or classes of, e.g., tumors.

As used herein, the term “**epitope**” has its ordinary meaning of a site on an antigen recognized by an antibody. Epitopes are typically segments of amino acids which are a small portion of the whole protein. Epitopes may be conformational (*i.e.*, discontinuous). That is, they may be formed from amino acids encoded by noncontiguous parts of a primary sequence that have been juxtaposed by protein

folding.

The terms "**favorable prognosis**" and "**unfavorable prognosis**" are known in the art. In the context of cancers, "favorable prognosis" means that there is a likelihood of tumor regression or longer survival times for patients with a favorable prognosis relative to those with unfavorable prognosis, whereas "unfavorable prognosis" means that the tumor is likely to be more aggressive, resulting in a poor outcome or a more rapid course of disease progression for the patient.

As used herein, the term "**fusion protein**," refers to a composite protein, i.e., a single contiguous amino acid sequence, made up of two (or more) distinct, heterologous polypeptides which are not normally fused together in a single amino acid sequence. Thus, a fusion protein may include a single amino acid sequence that contains two entirely distinct amino acid sequences or two similar or identical polypeptide sequences, provided that these sequences are not normally found together in a single amino acid sequence. Fusion proteins may generally be prepared using either recombinant nucleic acid methods, i.e., as a result of transcription and translation of a recombinant gene fusion product, which fusion comprises a segment encoding a polypeptide of the invention and a segment encoding a heterologous protein, or by chemical synthesis methods well known in the art. The non-hTERT region(s) of the fusion protein can be fused to the amino terminus of the hTERT polypeptide, the carboxy terminus, or both.

As used herein, the term "**gene product**" refers to an RNA molecule transcribed from a gene, or a protein encoded by the gene or translated from RNA.

As used herein, "**hTR**" (**human telomerase RNA**) refers to the RNA component of human telomerase and any naturally occurring alleles and variants or recombinant variants. hTR is described in detail in U.S. Patent No. 5,583,016 which is incorporated herein by reference in its entirety and for all purposes.

As used herein, the term "**immortal**," when referring to a cell, has its normal meaning in the telomerase art and refers to cells are those that have apparently unlimited replicative potential. Immortal can also refer to cells with increased proliferative capacity relative to their unmodified counterparts. Examples of immortal human cells are malignant tumor cells, germ line cells, and certain transformed human cell lines cultured *in vitro* (e.g., cells that have become immortal following transformation by viral oncogenes). In contrast, most normal human somatic cells are mortal, i.e., have limited replicative potential and become senescent after a finite number of cell divisions.

As used herein, the terms "**immunogen**" and "**immunogenic**" have their ordinary meaning in the art, *i.e.*, an immunogen is a molecule, such as a protein or other antigen, that can elicit an adaptive immune response upon injection into a person or an animal.

As used herein, the terms "**nucleic acid**" and "**polynucleotide**" are used interchangeably. Use of the term "polynucleotide" is not intended to exclude oligonucleotides (i.e., short polynucleotides) and can also refer to synthetic and/or non-naturally occurring nucleic acids (i.e., comprising nucleic acid analogues or modified backbone residues or linkages).

As used herein "**oligonucleotides**" or "**oligomers**" refer to a nucleic acid sequence of approximately 7 nucleotides or greater, and as many as approximately 100 nucleotides, which can be used as a probe or amplimer. Oligonucleotides are often between about 10 and about 50 nucleotides in length, more often between about 14 and about 35 nucleotides, very often between about 15 and about 25 nucleotides and can also refer to synthetic and/or non-naturally occurring nucleic acids (i.e., comprising nucleic acid analogues or modified backbone residues or linkages)..

As used herein, the term "**operably linked**," refers to a functional

relationship between two or more nucleic acid (e.g., DNA) segments: for example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence in an appropriate host cell or other expression system. Generally, sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be located in close proximity to the coding sequences whose transcription they enhance.

As used herein, the term "**polypeptide**" is used interchangeably herein with the term "protein," and refers to a polymer composed of amino acid residues, including synthetic, naturally-occurring and non-naturally occurring analogs thereof. Peptides are examples of polypeptides.

As used herein, a "**probe**" refers to a molecule that specifically binds another molecule. One example of a probe is a "**nucleic acid probe**" that specifically binds (i.e., anneals or hybridizes) to a substantially complementary nucleic acid. Another example of a probe is an "**antibody probe**" that specifically binds to a corresponding antigen or epitope.

As used herein, "**recombinant**" refers to a polynucleotide synthesized or otherwise manipulated *in vitro* (e.g., "recombinant polynucleotide"), to methods of using recombinant polynucleotides to produce gene products in cells or other biological systems, or to a polypeptide ("recombinant protein") encoded by a recombinant polynucleotide.

As used herein, a "**selection system**," in the context of stably transformed cell lines, refers to a method for identifying and/or selecting cells containing a recombinant nucleic acid of interest. A large variety of selection systems are known for identification of transformed cells and are suitable for use with the present invention. For example, cells transformed by plasmids or other vectors can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such



as the well known amp, gpt, neo and hyg genes, or other genes such as the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223-32 [1977]) and adenine phosphoribosyltransferase (Lowy et al., Cell 22:817 [1980]) genes which can be employed in tk- or apt- cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, *dhfr* which confers resistance to methotrexate and is also useful for gene amplification (Wigler et al., *Proc. Natl. Acad. Sci.*, 77:3567 [1980]); *npt*, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin et al., *J. Mol. Biol.*, 150:1 [1981]) and *als* or *pat*, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, in McGraw Hill Yearbook of Science and Technology, McGraw Hill, New York NY, pp 191-196, [1992]). Additional selectable genes have been described, for example, hygromycin resistance-conferring genes, *trpB*, which allows cells to utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman and Mulligan, *Proc. Natl. Acad. Sci.*, 85:8047 [1988]). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes et al., *Meth. Mol. Biol.*, 55:121 [1995]).

As used herein, the "sequence" of a gene (unless specifically stated otherwise), nucleic acid, protein, or peptide refers to the order of nucleotides in either or both strands of a double-stranded DNA molecule, e.g., the sequence of both the coding strand and its complement, or of a single-stranded nucleic acid molecule, or to the order of amino acids in a peptide or protein.

As used herein, "specific binding" refers to the ability of one molecule, typically an antibody or polynucleotide, to contact and associate with another specific molecule even in the presence of many other diverse molecules. For example, a single-stranded polynucleotide can specifically bind to a single-stranded polynucleotide that is

complementary in sequence, and an antibody specifically binds to (or "is specifically immunoreactive with") its corresponding antigen.

As used herein, "stringent hybridization conditions" or "stringency" refers to conditions in a range from about 5°C to about 20°C or 25°C below the melting temperature ( $T_m$ ) of the target sequence and a probe with exact or nearly exact complementarity to the target. As used herein, the melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the  $T_m$  of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) METHODS IN ENZYMOLOGY, VOL. 152: GUIDE TO MOLECULAR CLONING TECHNIQUES, San Diego: Academic Press, Inc. and Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory hereinafter, "Sambrook"), both incorporated herein by reference). As indicated by standard references, a simple estimate of the  $T_m$  value may be calculated by the equation:  $T_m = 81.5 + 0.41(\% G + C)$ , when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, *Quantitative Filter Hybridisation* in NUCLEIC ACID HYBRIDISATION (1985)). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of  $T_m$ . The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, and the like), and the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art, e.g., Sambrook, *supra* and Ausubel et al. *supra*. Typically, stringent hybridization conditions are salt concentrations less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion at pH 7.0 to 8.3, and temperatures at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). As noted, stringent conditions may also be achieved with

the addition of destabilizing agents such as formamide, in which case lower temperatures may be employed.

As used herein, the term "**substantial sequence identity**," in the context of nucleic acids, refers to a measure of sequence similarity between two polynucleotides. Substantial sequence identity can be determined by hybridization under stringent conditions, by direct comparison, or other means. For example, two polynucleotides can be identified as having substantial sequence identity if they are capable of specifically hybridizing to each other under stringent hybridization conditions. Other degrees of sequence identity (e.g., less than "substantial") can be characterized by hybridization under different conditions of stringency. Alternatively, substantial sequence identity can be described as a percentage identity between two nucleotide (or polypeptide) sequences. Two sequences are considered substantially identical when they are at least about 60% identical, preferably at least about 70% identical, or at least about 80% identical, or at least about 90% identical, or at least about 95% or 98% to 100% identical. Percentage sequence (nucleotide or amino acid) identity is typically calculated by determining the optimal alignment between two sequences and comparing the two sequences. For example an exogenous transcript used for sense suppression can be described as having a certain percentage of identity or similarity compared to a reference sequence (e.g., the corresponding endogenous sequence). Optimal alignment of sequences may be conducted using the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by inspection. The best alignment (i.e., resulting in the highest percentage of identity) generated by the various methods is selected. Typically these algorithms compare the two sequences over a "comparison window" (usually at least 18 nucleotides in length) to identify and

compare local regions of sequence similarity, thus allowing for small additions or deletions (i.e., gaps). Additions and deletions are typically 20 percent or less of the length of the sequence relative to the reference sequence, which does not comprise additions or deletions. It is sometimes desirable to describe sequence identity between two sequences in reference to a particular length or region (e.g., two sequences may be described as having at least 95% identity over a length of at least 500 basepairs). Usually the length will be at least about 50, 100, 200, 300, 400 or 500 basepairs, amino acids, or other residues. The percentage of sequence identity is calculated by comparing two optimally aligned sequences over the region of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, or U) occurs in both sequences to yield the number of matched positions, and determining the number (or percentage) of matched positions as compared to the total number of bases in the reference sequence or region of comparison. Alternatively, another indication that two nucleic acid sequences are similar is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

As used herein, the terms "**substantial identity**" or "**substantial similarity**" in the context of a polypeptide, refers to a degree of similarity between two polypeptides in which a polypeptides comprises a sequence with at least 70% sequence identity to a reference sequence, or 80%, or 85% or up to 100% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. Amino acid sequence similarity, or sequence identity, is determined by optimizing residue matches, if necessary, by introducing gaps as required. See Needleham et al. (1970) *J. Mol. Biol.* 48: 443-453; Sankoff et al. (1983) *Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison* Chapter One, Addison-Wesley, Reading, MA; and software packages from IntelliGenetics, Mountain View, CA; and the University of Wisconsin Genetics Computer Group, Madison, WI. As will be apparent to one of skill, the terms "substantial identity", "substantial similarity" and "substantial sequence identity" can be

used interchangeably with regard to polypeptides or polynucleotides.

As used herein, the term “**substantially pure**,” or “**substantially purified**,” when referring to a composition comprising a specified reagent, such as an antibody (e.g. an anti-hTERT antibody) is when at least about 75%, or at least about 90%, or at least about 95%, or at least about 99% or more of the specified reagent, for example, the immunoglobulin molecules present in a preparation that specifically binds an hTERT polypeptide.

As used herein, “**isolated**,” when referring to a molecule or composition, such as, for example, an RNP, means that the components of the RNP (e.g., at least one protein and at least one RNA) are separated from at least one other compound, such as a protein, other RNAs, or other contaminants with which they are associated *in vivo*. Thus, an RNP is considered isolated when the RNP has been isolated from, e.g., cell membrane, as in a cell extract. An isolated composition can, however, also be substantially pure.

As used herein, a “**telomerase negative**” cell is one in which telomerase is not expressed, i.e., no telomerase catalytic activity can be detected using a conventional assay or a TRAP assay for telomerase catalytic activity. As used herein, a “**telomerase positive**” cell is a cell in which telomerase is expressed (i.e. telomerase activity can be detected).

As used herein, a “**telomerase-related**” disease or condition is a disease or condition in a subject that is correlated with an abnormally high level of telomerase activity in cells of the individual, which can include any telomerase activity at all for most normal somatic cells, or which is correlated with a low level of telomerase activity that results in impairment of a normal cell function (e.g., fibroblast function in wound healing). Examples of telomerase-related conditions include, e.g., cancer (high telomerase activity in malignant cells) and infertility (low telomerase activity in germ-line cells).

**XI. EXAMPLES**

5 The following examples are provided to illustrate the present invention,  
and not by way of limitation.

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The following examples are provided to illustrate the present invention,  
and not by way of limitation.

10

**EXAMPLE 1****ISOLATION OF hTERT cDNA CLONES**

The following example details the isolation of hTERT and *S. pombe*  
telomerase cDNA.

***Background***

15

While telomerase RNA subunits have been identified in ciliates, yeast  
and mammals, protein subunits of the enzyme have not been identified as such prior to  
the present invention. Purification of telomerase from the ciliated protozoan *Euplotes*  
*aediculatus* yielded two proteins, termed p123 and p43 (Lingner (1996) *Proc. Natl.*  
*Acad. Sci. U.S.A.* 93:10712). *Euplotes aediculatus* is a hypotrichous ciliate having a  
20 macronuclei containing about  $8 \times 10^7$  telomeres and about  $3 \times 10^5$  molecules of  
telomerase. After purification, the active telomerase complex had a molecular mass of  
about 230 kD, corresponding to a 66 kD RNA subunit and two proteins of about 123  
kD and 43 kD (Lingner (1996) *supra*). Photocross-linking experiments indicated that  
the larger p123 protein was involved in specific binding of the telomeric DNA substrate  
25 (Lingner, (1996) *supra*).

The p123 and p43 proteins were sequenced and the cDNA clones which  
encoded these proteins were isolated. These *Euplotes* sequences were found to be  
unrelated to the *Tetrahymena* telomerase-associated proteins p80 and p95. Sequence  
analysis of the *Euplotes* p123 revealed reverse transcriptase (RT) motifs. Furthermore,  
30 sequence analysis of the *Euplotes* p123 revealed a yeast homolog, termed Est2 protein

(Lingner (1997) *Science* 276:561). Yeast Est2 had previously been shown to be essential for telomere maintenance *in vivo* (Lendvay (1996) *Genetics* 144:1399) but had not been identified as a telomerase catalytic protein. Site-specific mutagenesis demonstrated that the RT motifs of yeast Est2 are essential for telomeric DNA synthesis *in vivo* and *in vitro* (Lingner (1997) *supra*).

### ***Identifying and Characterizing S. pombe Telomerase***

PCR amplification of *S. pombe* DNA was carried out with degenerate sequence primers designed from the *Euplotes* p123 RT motifs (primer pair SEQ ID NO:\_\_\_ and SEQ ID NO:\_\_\_). Of the four prominent PCR products generated, a 120 base pair band encoded a peptide sequence homologous to p123 and Est2. This PCR product was used as a probe in colony hybridization and identified two overlapping clones from an *S. pombe* genomic library and three from an *S. pombe* cDNA library. Sequence analysis revealed that none of the three *S. pombe* cDNA clones was full length, so reverse transcriptase (RT)-PCR was used to obtain the sequences encoding the protein's N-terminus.

Complete sequencing of these clones revealed a putative *S. pombe* telomerase RT gene, *trt1* (SEQ ID NO:\_\_\_). The complete nucleotide sequence of *trt1* has been deposited in GenBank, Accession number \_\_\_\_\_. Analysis of the sequence showed that *trt1* encoded a basic protein with a predicted molecular mass of 116 kD. It was found that homology with p123 and Est2 was especially high in the seven reverse transcriptase motifs, underlined and designated as motifs 1, 2, A, B, C, D, and E (see Figure 1). An additional telomerase-specific motif was found, designated the T-motif. Fifteen introns, ranging in size from 36 to 71 base pairs, interrupted the coding sequence.

To test *S. pombe trt1* (SEQ ID NO:\_\_\_) as a catalytic subunit, two deletion constructs were created. One removed only motifs B through D in the RT domains (SEQ ID NO:\_\_\_). The second removed 99% of the open reading frame (SEQ ID NO:\_\_\_).

Haploid cells grown from *S. pombe* spores of both mutants showed progressive telomere shortening to the point where hybridization to telomeric repeats

became almost undetectable. A *trt1<sup>+</sup>/trt1<sup>-</sup>* diploid was sporulated and the resulting tetrads were dissected and germinated on a yeast extract medium supplemented with amino acids (a YES plate, Alfa, (1993) *Experiments with Fission Yeast*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Colonies derived from each spore  
 5 were grown at 32°C for three days, and streaked successively to fresh YES plates every three days. A colony from each round was placed in six ml of YES liquid culture at 32°C and grown to stationary phase. Genomic DNA was prepared. After digestion with *Apa*I, DNA was subjected to electrophoresis on a 2.3% agarose gel, stained with ethidium bromide to confirm approximately equal loading in each lane, then transferred  
 10 to a nylon membrane and hybridized to a telomeric DNA probe.

Senescence was indicated by the delayed onset of failure to grow on agar (typically at the fourth streak-out after germination) and by colonies with increasingly ragged edges (colony morphology shown in Figure 22B) and by increasingly high fractions of elongated cells (as shown in Figure 22C). Cells were plated on Minimal  
 15 Medium (Alfa (1993) *supra*) with glutamic acid substituted for ammonium chloride for two days at 32°C prior to photography.

When individual enlarged cells were separated on the dissecting microscope, the majority were found to undergo no further division. The same telomerase negative (*trt1<sup>-</sup>*) cell population always contained normal-sized cells which  
 20 continued to divide, but which frequently produced non-dividing progeny. The telomerase-negative survivors may use a recombinational mode of telomere maintenance as documented in budding yeast strains that have various telomere-replication genes deleted (Lendvay (1996) *supra*, Lundblad (1993) *Cell* 73:347).



*Identifying and Characterizing Human Telomerase*

An EST (expressed sequence tag) derived from human telomerase reverse transcriptase (hTERT) cDNA was identified by a BLAST search of the dbEST (expressed sequence tag) Genbank database, designated Genbank AA28196 (SEQ ID NO: \_\_) using the \_\_\_\_\_. The AA281296 EST is 389 nucleotides long and its residue positions in hTERT cDNA clone (SEQ ID NO: \_\_) are from residues 1679 to 2076. A clone containing the EST sequence, designated clone #712562 (SEQ ID NO: \_\_), was obtained from the I.M.A.G.E. Consortium (Human Genome Center, DOE, Lawrence Livermore National Laboratory, Livermore, CA). This clone was obtained from a cDNA library of germinal B cells derived by flow sorting of tonsil cells. Complete sequencing of this hTERT cDNA clone (SEQ ID NO: \_\_) showed all eight telomerase RT (TRT) motifs, as shown in Figure 1. However, this hTERT clone did not encode a contiguous portion of a TRT because RT motifs B', C, D, and E, were contained in a different open reading frame than the more N-terminal RT motifs. In addition, the distance between RT motifs A and B was substantially shorter than that of the three previously known (non-human) TRTs.

To isolate a full length cDNA clone, a cDNA library derived from the human 293 cell line (described above) which expresses high levels of telomerase activity, was screened. A lambda cDNA library from the 293 cell line was partitioned into 25 pools containing about 200,000 plaques each. Each pool was screened by PCR with the primer pair 5'-CGGAAGAGTGTCTGGAGCAA-3' (SEQ ID NO: \_\_) and 5'-GGATGAAGCGGAGTCTGGA-3' (SEQ ID NO: \_\_). Six subpools of one positive primary pool were further screened by PCR using this same primer pair. For both the primary and the secondary subpool screening, hTERT was amplified for a total of 31 cycles at: 94°C, 45 seconds; 60°C, 45 seconds; and 72°C, 90 seconds. As a control, RNA of the house-keeping enzyme GAPDH was amplified using the primer pair 5'-CTCAGACACCATGGGGAAGGTGA-3' (SEQ ID NO: \_\_) and 5'-ATGATCTTGAGGCTGTTGTCATA-3' (SEQ ID NO: \_\_) for a total of 16 cycles at 94°C, 45 seconds; 55°C, 45 seconds; and 72°C, 90 seconds.

One hTERT positive subpool from the secondary screening was then

screened by plaque hybridization with a probe from the 5' region of clone #712562 (SEQ ID NO: \_\_). One phage was positively identified (designated Lambda phage 25-1.1, ATCC 209024, deposited May 12, 1997). It contained an approximately four kilobase insert, which was excised and subcloned into the EcoRI site of pBluescript II SK+ vector (Stratagene, San Diego, CA) as an EcoRI fragment. This cDNA clone-  
 5 containing plasmid was designated pGRN121. The cDNA insert totals 7029 base pairs (SEQ ID NO:1). The complete nucleotide sequence of pGRN121 has been deposited in Genbank (accession # \_\_\_\_\_) and with the ATCC, given the accession no. ATCC 209016, deposited May 6, 1997.

10

## EXAMPLE 2

### CORRELATION OF hTERT ABUNDANCE AND CELL IMMORTALITY

The relative abundance of hTERT mRNA was assessed in six telomerase-negative mortal cell strains and six telomerase-positive immortal cell lines (**Figure 5**).

15 The steady state level of hTERT mRNA was significantly increased (\*27) in immortal cell lines that had previously been shown to have active telomerase (\*7). Lower levels of the hTERT mRNA were detected in some telomerase-negative cell strains.

RT-PCR for hTERT, hTR, TP1 (telomerase-associated protein related to *Tetrahymena* p80 [Harrington et al., 1997, *Science* 275:973; Nakayama et al., 1997, *Cell* 88:875]) and GAPDH (to normalize for equal amounts of RNA template) was  
 20 carried out on RNA derived from the following cells: (1) human fetal lung fibroblasts GFL, (2) human fetal skin fibroblasts GFS, (3) adult prostate stromal fibroblasts 31 YO, (4) human fetal knee synovial fibroblasts HSF, (5) neonatal foreskin fibroblasts BJ, (6) human fetal lung fibroblasts IMR90, and immortalized cell lines: (7) melanoma  
 25 LOX IMVI, (8) leukemia U251, (9) NCI H23 lung carcinoma, (10) colon adenocarcinoma SW620, (11) breast tumor MCF7, (12) 293 adenovirus E1 transformed human embryonic kidney cell line.

hTERT nucleic acid was amplified from cDNA using oligonucleotide primers LT5 and LT6 (**Table 2**) for a total of 31 cycles (94°C 45s, 60°C 45s, 72°C  
 30 90s). GAPDH was amplified using primers KI36

(CTCAGACACCATGGGGAAGGTGA) and K137  
(ATGATCTTGAGGCTGTTGTCATA) totals 16 cycles (94°C 45 s, 55°C 45 s, 72°C  
90 s). hTR was amplified using primers F3b  
(TCTAACCCTAACTGAGAAGGGCGTAG) and R3c  
5 (GTTTGCTCTAGAATGAACGGTGAAG) for a total of 22 cycles (94 °C 45s, 55  
°C 45 s, 72 °C 90s). TP1 mRNA was amplified using primers TP1.1 and TP1.2 for 28  
cycles (cycles the same as hTRT). Reaction products were resolved on an 8%  
polyacrylamide gel, stained with SYBR Green (Molecular Probes) and visualized by  
scanning on a Storm 860 (Molecular Dynamics). The results, shown in Figure 5,  
10 demonstrate that hTRT mRNA levels correlate directly with telomerase activity levels  
in the cells tested.

### EXAMPLE 3

#### CHARACTERATION OF AN hTRT INTRONIC SEQUENCE

15 A putative intron was first identified by PCR amplification of human  
genomic DNA, as described in this example, and subsequently confirmed by  
sequencing the genomic clone λGϕ5 (see **Example 4**). PCR amplification was carried  
out using the forward primer TCP1.57 paired individually with the reverse primers  
TCP1.46, TCP1.48, TCP1.50, TCP1.52, TCP1.54, TCP1.56, and TCP1.58 (see **Table**  
20 **2**). The products from genomic DNA of the TCP1.57/TCP1.46, TCP1.48, TCP1.50,  
TCP1.52, TCP1.54, or TCP1.56 amplifications were approximately 100 basepairs  
larger than the products of the pGRN121 amplifications. The TCP1.57/TCP1.58  
amplification was the same on either genomic or pGRN121 DNA. This indicated the  
genomic DNA contained an insertion between the sites for TCP1.58 and TCP1.50. The  
25 PCR products of TCP1.57/TCP1.50 and TCP1.57/TCP1.52 were sequenced directly,  
without subcloning, using the primers TCP1.39, TCP1.57, and TCP1.49.

As shown below, the 104-base intronic sequence (**SEQ ID NO: 7**) is  
inserted in the hTRT mRNA (shown in bold) at the junction corresponding to bases 274  
and 275 of **SEQ. ID. NO: 1**:

30

CCCCCGCCGCCCCCTCCTTCCGCCAG/GTGGGCCTCCCCGGGGTCGGCG  
 TCCGGCTGGGGTTGAGGGCGGCCGGGGGAACCAGCGACATGCGGAGAG  
 CAGCGCAGGCGACTCAGGGCGCTTCCCCCGCAG/GTGTCCTGCCTGAAGG  
 AGCTGGTGGCCCGAGTGCTGCAG

5

The “/” indicates the splice junctions; the sequence shows good matches to consensus 5' and 3' splice site sequences typical for human introns.

This intron contains motifs characteristic of a topoisomerase II cleavage site and a NFκB binding site (see **Figure 21**). These motifs are of interest, in part, because expression of topoisomerase II is up regulated in most tumors. It functions to relax DNA by cutting and rewinding the DNA, thus increasing expression of particular genes. Inhibitors of topoisomerase II have been shown to work as anti-tumor agents. In the case of NFκB, this transcription factor may play a role in regulation of telomerase during terminal differentiation, NFκB may play a role in early repression of telomerase during development and so is another target for therapeutic intervention to regulate telomerase activity in cells.

15

#### EXAMPLE 4

20

#### CLONING OF LAMBDA PHAGE GΦ5 AND CHARACTERIZATION OF hTRT GENOMIC SEQUENCES

##### a) Lambda GΦ5

25

A human genomic DNA library was screened by PCR and hybridization to identify a genomic clone containing hTRT RNA coding sequences. The library was a human fibroblast genomic library made using DNA from WI38 lung fibroblast cells (Stratagene, Cat # 946204). In this library, partial Sau3AI fragments are ligated into the XhoI site of Lambda FIX®II Vector (Stratagene), with an insert size of 9-22 kb.

30

The genomic library was divided into pools of 150,000 phage each, and each pool screened by nested PCR (outer primer pair TCP1.52 & TCP1.57; inner pair TCP1.49 & TCP1.50, see **Table 1**). These primer pairs span a putative intron (see **Example 3, supra**) in the genomic DNA of hTRT and ensured the PCR product was

derived from a genomic source and not from contamination by the hTRT cDNA clone. Positive pools were further subdivided until a pool of 2000 phage was obtained. This pool was plated at low density and screened via hybridization with a DNA fragment encompassing basepairs 1552-2108 of **SEQ. ID. NO. 1** (restriction sites SphI and EcoRV, respectively).

Two positive clones were isolated and rescreened via nested PCR as described above; both clones were positive by PCR. One of the clones ( $\lambda$ G $\Phi$ 5) was digested with NotI, revealing an insert size of approximately 20 kb. Subsequent mapping (see below) indicated the insert size was 15 kb and that phage G $\Phi$ 5 contains approximately 13 kb of DNA upstream from the start site of the cDNA sequence.

Phage G $\Phi$ 5 was mapped by restriction enzyme digestion and DNA sequencing. The resulting map is shown in **Figure 7**. The phage DNA was digested with *Nco*I and the fragments cloned into pBBS167. The resulting subclones were screened by PCR to identify those containing sequence corresponding to the 5' region of the hTRT cDNA. A subclone (pGRN140) containing a 9 kb *Nco*I fragment (with hTRT gene sequence and 4-5 kb of lambda vector sequence) was partially sequenced to determine the orientation of the insert. pGRN 140 was digested using *Sal*I to remove lambda vector sequences, resulting in pGRN144. pGRN144 was then sequenced. The sequence is provided in **Seq. ID. NO: 6**. The 5' end of the hTRT mRNA corresponds to base \_\_\_\_\_ of **Seq. ID. NO: 6**. As indicated in **Figure 7**, two Alu sequence elements are located 1700 base pairs upstream of the hTRT cDNA 5' end and provide a likely upstream limit to the promoter region of hTRT. The sequence also reveals an intron positioned at bases \_\_\_\_\_ **Seq. ID. NO: 6**, 3' to the intron described in **Example 3, supra**.

#### b) Additional genomic clones

In addition to the genomic clone described above, two P1 bacteriophage clones and one human BAC clone are provided as illustrative embodiments of the invention. P1 inserts are usually 75-100 kb, and BAC inserts are usually over 100 Kb.

The P1 clones (DMPC-HFF#1-477(F6) -GS #15371 and

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DMPC-HEF#1-1103(H6) -GS #15372) were obtained by PCR screening of a human P1 library derived from human foreskin fibroblast cells (Shepherd et al., 1994, *PNAS USA* 91:2629) using primers TCP1.12 and UTR2 which amplify the 3' end of hTRT. These clones were both negative (failed to amplify) with primers that amplify the 5' end of

5 hTRT.

The human BAC clone (326 E 20) was obtained with a hybridization screen of a BAC human genomic library using an 1143 bp Sph1/Xmn1 fragment of **SEQ. ID. NO: 1** (bases 1552-2695) that encompasses the RT motif region. The clone is believed to include the 5'. The hTRT genomic clones in this example are believed to

10 encompass the entire hTRT gene.

## EXAMPLE 5

**CHROMOSOMAL LOCATION OF HTRT GENE**

The hTRT gene was localized to chromosome 5p by radiation hybrid mapping (Boehnke et al., 1991, *Am J Hum Genet* 49:1174; Walter et al., 1994, *Nature Genet* 7:22) using the medium resolution Stanford G3 panel of 83 RH clones of the whole human genome (created at the Stanford Human Genome Center). A human lymphoblastoid cell line (donor; rM) was exposed to 10,000 rad of x-rays and was then fused with nonirradiated hamster recipient cells (A3). Eighty-three independent somatic cell hybrid clones were isolated, and each represents a fusion event between an irradiated donor cell and a recipient hamster cell. The panel of G3 DNA was used for ordering markers in the region of interest as well as establishing the distance between these markers.

The primers used for the RH mapping were TCP1.12 and UTR2 with amplification conditions of 94°C 45 sec, 55°C 45 sec, 72°C 45 sec, for 45 cycles using Boehringer Mannheim Taq buffer and Perkin-Elmer Taq. The 83 pools were amplified independently and 14 (17%) scored positive for hTRT (by appearance of a 346 bp band). The amplification results were submitted to Stanford RH server, which then provided the map location, 5p, and the closest marker, STS D5S678.

By querying the Genethon genome mapping web site, the map location identified a YAC that contains the STS marker D5S678: CEPH YAC 780\_C\_3 Size: 390,660kb. This YAC also contained chromosome 17 markers. This result indicated that the hTRT gene is on chromosome 5, near the telomeric end. There are increased copy numbers of 5p in a number of tumors. Cri-du-chat syndrome also has been mapped to deletions in this region.

## EXAMPLE 6

**DESIGN AND CONSTRUCTION OF VECTORS FOR EXPRESSION OF HTRT PROTEINS AND POLYNUCLEOTIDES****Expression of hTRT in Bacteria**

The following example details the design of hTRT-expressing bacterial

expression vectors to produce large quantities of full-length, biologically active hTERT (SEQ ID NO: 2). Generation of biologically active hTERT protein in this manner is useful for telomerase reconstitution assays, assaying for telomerase activity modulators, analysis of the activity of newly isolated species of hTERT, identifying and isolating compounds which specifically associate with hTERT, analysis of the activity of an hTERT variant protein that has been site-specifically mutated, as described above, and as an immunogen, as a few examples.

***pThioHis A/hTERT Bacterial Expression Vector***

To produce large quantities of full-length hTERT (SEQ ID NO:2), the bacterial expression vector pThioHis A (Invitrogen, San Diego, CA) was selected as an expression system. The hTERT-coding insert includes nucleotides 707 to 4776 of the hTERT insert in the plasmid pGRN121 (SEQ ID NO:1). This nucleotide sequence includes the complete coding sequence for hTERT protein (SEQ ID NO:2).

This expression vector of the invention is designed for inducible expression in bacteria. The vector can be induced to express, in *E. coli*, high levels of a fusion protein composed of a cleavable, HIS tagged thioredoxin moiety and the full length hTERT protein. The use of the expression system was in substantial accordance with the manufacturer's instructions. The amino acid sequence of the fusion protein encoded by the resulting vector of the invention is shown below; (-\*-) denotes an enterokinase cleavage site (SEQ ID NO: \_\_):

MSDKIIHLTDDSFDTDLKADGAILVDFWAHWCGPCKMIAPILDEIADEYQ  
GKLTIVAKLRIDHNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEF  
LDANLAGSGSGDDDDK - \*-VPMHELEIFEFAAASTQRCVLLRTWEALAPATP  
AMPRAPRCRAVRSLLRSHYREVLPLATFVRRLLGPQGWRLVQRGDPAEFR  
ALVAQCLVCVPWDARPPPAAPSFRQVSCLKELVARVLQRLCERGAKNVL  
AFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLA  
RCALFVLVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAG  
VPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDR  
GFCVVSPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVY  
AETKHFLYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRL  
PQRYWQMRPLFLELLGNHAQCYPGVLLKTHCPLRAAVTPAAGVCAREKPGQSVAAP  
EEDTDPRLVQLLRQHSSPWQVYGFVRACLRLVPPGLWGSRHNERFLRNTKKFIS  
LGKHAKLSLQELTWKMSVRDCAWLRRSPGVGCVPAAEHLREEILAKFLHWLMSVYV  
VELLRSFYVTETTFQKNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHR



EARPALLTSRLRFIPKPDGLRPIVNM DYVVGARTFRREKRAERLTSRVKALFSVLNY  
 ERARRPGLLGASVLGLDDIHRWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDR  
 LTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQ  
 5 ETSPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYVQCQGIPOGSILS  
 TLLCSLCYGD MENKLFAGIRRDG LLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYG  
 CVVNLRKTVVNF PVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYART  
 SIRASLTFNRGFKAGRNMRRKLF GVLRLKCHSLFLDLQVNSLQTVCTNIYKILLQA  
 YRFHACVLQLPFHQVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLP  
 10 SEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGTTLTALEAAANPAL  
 PSDFKTILD

# pGEX-2TK with hTRT Nucleotides 3272 to 4177 of pGRN121

This construct of the invention is used to produce fusion protein for, e.g.,  
 the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Fragments  
 of hTRT can also be used for other purposes, such as to modulate telomerase activity, for  
 example, as a dominant mutant or to prevent the association of telomerase with other  
 proteins or nucleic acids.

To produce large quantities of an hTRT protein fragment, the *E. coli*  
 20 expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) was selected, and used  
 essentially according to manufacturer's instructions to make an expression vector of the  
 invention. The resulting construct contains an insert derived from nucleotides 3272 to  
 4177 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO: \_\_) . The vector directs  
 expression in *E. coli* of high levels of a fusion protein composed of  
 25 glutathione-S-transferase sequence (underlined below), thrombin cleavage sequence (double  
 underlined), recognition sequence for heart muscle protein kinase (italicized), residues  
 introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold) (SEQ  
 ID NO: 38) as shown below:

30 MSPILGYWKIKGLVOPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPY  
YIDGDVKLTOSMAIRYIADKHNMLGGCPKERAESMLEGAVLDIRYGVSRIAYSSKD  
FETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLD  
AFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATFGGGDHPPKSDLVPRGSRR  
 ASV[GSVTK] **IPQGSILSTLLCSLCYGD MENKLFAGIRRDG LLLRLVDDFLLVTPHL**  
**THAKTFLRTLVRGVPEYGC**CVVNLRKTVVNF PVEDEALGGTAFVQMPAHGLFPWCGLL  
 35 LDTRTLEVQSDYSSYARTSIRASVTFNRGFKAGRNMRRKLF GVLRLKCHSLFLDLQV

NSLQTVCTNIYKILLQAYRFHACVLQLPFHQVWKNPTFFLRVISDTASLCYSILK  
 AKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLKLTRHRVTYVPLLGSLRTAQTQLSR  
 KLP GTTTLTALEAAANPALPSDFKTILD

5 When this fusion protein was expressed, it formed insoluble aggregates. It was treated generally as described above, section entitled purification of proteins from inclusion bodies. Specifically, induced cells were suspended in PBS (20 mM sodium phosphate, pH 7.4, 150 mM NaCl) and disrupted by sonication. NP-40 was added to 0.1%, and the mixture was incubated for 30 minutes at 4°C with gentle mixing. The insoluble material was collected by centrifugation at 25,000g for 30 minutes at 4°C. The insoluble material was washed once in 4M urea in PBS, collected by centrifugation, then washed again in PBS. The collected pellet was estimated to contain greater than 75% fusion protein. This material was dried in a speed vacuum, then suspended in adjuvant for injection into mice and rabbits for the generation of antibodies. Separation of the recombinant protein from the glutathione S-transferase moiety is accomplished by site-specific proteolysis using thrombin according to manufacturer's instructions.

***pGEX-2TK with hTRT Nucleotides 2426 to 3274 of pGRN121, with HIS-8 Tag***

To produce large quantities of a fragment of hTRT, another *E. coli* expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) construct was prepared. This construct contains an insert derived from nucleotides 2426 to 3274 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1) and sequence encoding eight consecutive histidine residues (HIS-8 Tag). The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase sequence (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized), a set of three and a set of five residues introduced by cloning are in brackets ([GSV] and [GSVTK]) eight consecutive histidines (also double underlined) and hTRT protein fragment (in bold) (SEQ ID NO:39):

30 MSPILGYWKIKGLVOPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPY  
YIDGDVKLTOSMAIIRYIADKHNMLGGCPKERAETSMLEGAVLDIRYGVSR  
IAYSKD  
FETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDP  
MCLD  
AFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATFGGGDHPPKSDLVPRG  
SRR

ASV[GSV] HHHHHHHHGSVTKMSVYVVELLRSFFYVTETTFQKNRLFFYRPSVWSKL  
 QSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVNMDYVVG  
 ARTFRREKRAERLTSRVKALFVNLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVR  
 AQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHV  
 5 RKAFKSHVSTLTDLPYMRQFVAHLQETSPLRDAVVIEQSSSLNEASSGLFDVFLRF  
 MCHHAVRIRGKSYVQCQGI

This vector can be used to produce fusion protein for the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Additionally, this fusion protein can be used to affinity purify antibodies raised to hTRT peptides that are encompassed within the fusion protein. Separation of the recombinant protein from the glutathione S-transferase moiety can be accomplished by site-specific proteolysis using thrombin according to manufacturer's instructions.

15 *pGEX-2TK with hTRT Nucleotides 2426 to 3274 of pGRN121, no HIS-8 Tag*

To produce large quantities of a fragment of hTRT, another *E. coli* expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) construct was prepared. This vector construct can be used to produce fusion protein for the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Additionally, this fusion protein can be used to affinity purify antibodies raised to hTRT peptides that are in the fusion protein.

This construct contains an insert derived from nucleotides 2426 to 3274 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1), but without the HIS-8 tag. The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle protein kinase (italicized), residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold) (SEQ ID NO:40):

30 MSPILGYWKIKGLVOPTRLLLEYLEEKYEELHYERDEGDKWRNKKFEL  
GLEFPNLPYYIDGDVKLTOSMAIIRYIADKHNMMLGGCPKERAELISMLEGAVLDIRYG  
VSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVV  
LYMDPMCLDAFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATFGGGDHPKPS  
DLVPRGSRRASV[GSVTK] **MSVYVVELLRSFFYVTETTFQKNRLFFYRPSVWSKLQS**  
**IGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVNMDYVVGAR**  
**TFRREKRAERLTSRKALFVNLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVRAQD**

PPPEYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGVRKAFK  
SHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIEQSSSLNEASGLFDVFLRFMCHHAV  
RIRGKSYVQCQGI

5 Separation of the recombinant protein from the glutathione S-transferase moiety can be accomplished by site-specific proteolysis using thrombin according to manufacturer's instructions.

*pGEX-2TK with hTRT Nucleotides 1625 to 2458 of pGRN121*

10 To produce large quantities of a fragment of hTRT protein, another *E. coli* expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) construct was prepared. This vector construct can be used to produce fusion protein for the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Additionally, this fusion protein can be used to affinity purify antibodies raised to hTRT peptides that are in the fusion protein.

This construct contains an insert derived from nucleotides 1625 to 2458 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1). The vector directs expression in *E coli* of high levels of a fusion protein composed of glutathione-S-transferase, (underlined), thrombin cleavage sequence (double underlined), recognition sequence for 20 heart muscle protein kinase (italicized) residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold) (SEQ ID NO:41):

25 MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFEL  
GLEFPNLPYYIDGDVKLTQSMAIRYIADKHNMMLGGCPKERAIEISMLEGAVL  
DIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPD  
FMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLO  
GWQATFGGGDHPPKSDLVPRGSRRA[GSVTK]ATSLEGALSGTRHSHPSV  
GRQHHAGPPSTSRPPRPWDTPCPPVYAETKHFLYSSGDKEQLRPSFLLSSL  
RPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGN  
30 HAQCPYGVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRL  
VQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSRHNERFLRNTKKFISL  
GKHAKLSLQELTWKMSVRDCAWLRRSPGVGCVPAAEHRLREEILAKFL  
HWLMSVYVVELLRS

35 Separation of the recombinant protein from the glutathione S-transferase

moiety is accomplished by site-specific proteolysis using thrombin according to manufacturer's instructions.

***pGEX-2TK with hTRT Nucleotides 782 to 1636 of pGRN121***

5 To produce large quantities of a fragment of hTRT protein, another *E. coli* expression vector pGEX-2TK (Pharmacia Biotech, Piscataway N.J) construct was prepared. This vector can be used to produce fusion protein for the purpose of raising polyclonal and monoclonal antibodies to hTRT protein. Additionally, this fusion protein can be used to affinity purify antibodies raised to hTRT peptides that are encompassed 10 within the fusion protein.

This construct contains an insert derived from nucleotides 782 to 1636 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1). The vector directs expression in *E. coli* of high levels of a fusion protein composed of glutathione-S-transferase, (underlined), thrombin cleavage sequence (double underlined), recognition sequence for heart muscle 15 protein kinase (italicized) residues introduced by cloning in brackets ([GSVTK]) and hTRT protein fragment (in bold) (SEQ ID NO:42):

20 MSPILGYWKIKGLVOPTRLILLEYLEEKYEEHLYERDEGDKWRNKKFEL  
GLEFPNLPYYIDGDVKLTOSMAIIRYIADKHNLGGCPKERAEISMLEGAVLDIRYG  
VSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDEFMLYDALDVV  
LYMDPMCCLDAFPKLVCFKKRIEAIPOIDKYLKSSKYIAWPLOGWOATFGGGDHPPKS  
DLVPRGSRRASV[GSVTK]MPRAPRCRAVRSLLSHYREVLPLATFVRRLGPGQWRLV  
 QRGDPAAFRALVAQCLVCVPWDARPPAAPSFQVSLKELVARVLQRLCERGAKNVL  
 AFGFALLDGARGGPPEATTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLAR  
 CALFVLVAPCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVP  
 25 LGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGF  
 CTVSPARPAEEATSL

Separation of the recombinant protein from the glutathione S-transferase moiety is accomplished by site-specific proteolysis using thrombin according to 30 manufacturer's instructions.

***pT7FLhTRT with hTRT cDNA Lacking 5'-Non-Coding Sequence***

As described above, in one embodiment, the invention provides for an hTRT

that is modified in a site-specific manner to facilitate cloning into bacterial, mammalian, yeast and insect expression vectors without any 5' untranslated hTRT sequence. In some circumstances, minimizing the amount of non-protein encoding sequence allows for improved protein production (yield) and increased mRNA stability.

5           This was effected by engineering an additional restriction endonuclease site just upstream (5') to the start (ATG) codon of the hTRT coding sequence SEQ ID NO:1. The creation of a restriction site just 5' to the coding region of the protein allows for efficient production of a wide variety of vectors that encode fusion proteins, such as fusion proteins comprising labels and peptide TAGs, for immunodetection and purification.

10           Specifically, the oligonucleotide 5'- CCGGCCACCCCCCATATGCCGCGCGTCCC-3' (SEQ NO: \_\_) was used as described above to modify hTRT cDNA nucleotides 779 to 781 (of SEQ ID NO:1) from GCG to CAT. These 3 nucleotides are the last nucleotides before the ATG start codon so they do not modify the protein sequence. The change in sequence results in the creation of 15 a unique NdeI restriction site in the hTRT cDNA. hTRT single-stranded DNA was used as a DNA source for the site directed mutagenesis. The resulting plasmid was sequenced to confirm the success of the mutagenesis.

          This modification allowed the construction of the following plasmid of the invention, designated pT7FLhTRT. The site-specifically modified hTRT sequence 20 (addition of the NdeI restriction site) was digested with NdeI and NotI (a restriction enzyme that generates blunt ended DNA) to generate an hTRT fragment. The fragment was then cloned into a pSL3418 plasmid previously restriction digested with NdeI and SmaI. pSL 3418 is a modified pAED4 plasmid into which a FLAG sequence (Immunex Corp, Seattle WA) and an enterokinase sequence are inserted just upstream from the above- 25 referenced NdeI site. This plasmid allows the expression of full length hTRT (with a Flag-Tag at its 5' end) in an *E.coli* strain expressing the T7 RNA polymerase.

### ***MPSV-hTRT Expression Plasmids***

          The invention also provides for a wide variety of expression systems for use 30 in expressing hTRT in mammalian cells to give high expression levels of recombinant

hTERT protein. The MPSV expression system is generally described by Lin, J-H (1994) *Gene* 47:287-292.

In this expression system, while the hTERT coding sequence (SEQ ID NO: \_\_) itself is unchanged, exogenous transcriptional control elements are incorporated into the vector. The myeloproliferative sarcoma virus (MPSV) LTR (MPSV-LTR) promoter, enhanced by the cytomegalovirus (CMV) enhancer, is incorporated for transcriptional initiation. This promoter consistently shows higher expression levels in cell lines (see Lin, J-H (1994) *supra*). A Kozak consensus sequence is incorporated for translation initiation (see Kozak (1996) *Mamm. Genome* 7:563-574). All extraneous 5' and 3' untranslated hTERT sequences have been removed from the resulting vector (designated pGRN133) of the invention to insure that these sequences do not interfere with expression, as discussed above. A control, hTERT "antisense" vector was also constructed. This vector (designated pGRN134) is identical to pGRN133, except that the hTERT insert is the antisense sequence of hTERT SEQ ID NO:1.

15 Two selection markers, PAC (Puromycin-N-acetyl-transferase = Puromycin resistance) and HygB (Hygromycin B = Hygromycin resistance) are present for selection of the plasmids after transfection (see discussion referring to selectable markers, above). Double selection using markers on both sides of the vector polylinker can increase the stability of the hTERT coding sequence. A DHFR (dihydrofolate reductase) encoding  
20 sequence is included to allow amplification of the expression cassette after stable clones are made. Other means of gene amplification can also be used to increase recombinant protein yields.

#### Expression of hTERT Telomerase in Yeast

25 The following example details the construction of hTERT-expressing yeast expression vectors of the invention to produce large quantities of full-length, biologically active hTERT (SEQ ID NO:2). Use of biologically active hTERT in the many embodiments of the invention is described above.

#### ***Pichia pastoris* Expression Vector pPICZ B and Full Length hTERT \$\$\$**

30 To produce large quantities of full-length, biologically active hTERT (SEQ ID

NO: \_\_), the *Pichia pastoris* expression vector pPICZ B (Invitrogen, San Diego, CA) was selected. The hTRT-coding sequence insert was derived from nucleotides 659 to 4801 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1). This nucleotide sequence includes the full-length sequence encoding hTRT (SEQ ID NO:2). This expression vector is designed for inducible expression in *P. pastoris* of high levels of full-length, unmodified hTRT protein (SEQ ID NO:2). Expression is driven by a yeast promoter, but the expressed sequence utilizes the hTRT initiation and termination codons. No exogenous codons were introduced by the cloning. The resulting pPICZ B/hTRT vector (Invitrogen, San Diego, CA) was used to transform the yeast.

#### 10 ***Pichia pastoris* Expression Vector hTRT-His6/pPICZ B**

A second *Pichia pastoris* expression vector of the invention derived from pPICZ B (Invitrogen, San Diego, CA), also contains the full-length sequence encoding hTRT (SEQ ID NO:2) derived from nucleotides 659 to 4801 of the hTRT insert in the plasmid pGRN121 (SEQ ID NO:1). This hTRT-His6/pPICZ B expression vector encodes full length hTRT protein (SEQ ID NO:2) fused at its C-terminus to the Myc epitope and His6 reporter sequences. The hTRT stop codon has been removed and replaced by vector sequences encoding the Myc epitope and the His6 reporter tag as well as a stop codon. This vector is designed to direct high-level inducible expression in yeast of the following fusion protein, which consists of hTRT sequence (underlined), vector sequences in brackets 20([L] and [NSAVD]) the Myc epitope (double underlined), and the His6 tag (italicized) (SEQ ID NO:45):

MPRAPRCRAVRSLLRSHYREVLPLATFVRR LGPOGWRLVORGDPAAFRAL  
 VAOCIVCVPWDARPPPAAPSFROVSCLKELVARVLORLCERGAKNVLA FG  
 FALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLV  
 25 HLLARCALEFVLVAPSCAYOVCGPPLYOLGAATOARPPPHASGPRRRLGCERA  
 WNHSVREAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGO  
 GSWAHPGRTRGPSDRGFCVVSPARPAEEATSLEGALSGTRHSHPSVGROHHAGPPST  
 SRPPRPWDTPCPPVYAETKHFYSSGDKEOLRPSFLLSSLRPSLTGARRLVETIFLG  
 SRPWPMPGTPRRLPRLPORYWOMRPLFLELLGNHAOCYPGVLLKTHCPLRAAVTPAAG  
 30 VCAREKPOGSVAAPPEEDTDPRRLVOLLROHSSPWVYGFVRACLRRLVPPGLWGSR  
 HNERRFLRNTKKFISLGKHAKLSLOELTWKMSVRDCAWLRRLSPGVGCVPAAEHLRE  
 EILAKFLHWLMSVYVVELLRSEFFYVTETTFOKNRLEFFYRKSVWSKLOSIGIROHLKR  
 VOLRELSEAEVROHREARPALLTSRLRFIPKPDGLRPIVNMDYVVGARTFERREKRAE  
 RLTSRVKALFSVLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVRAODPPPELYFV



KVDVTGAYDTIPODRLTEVIASIIK PONTYCVRRYAVVOKAAHGHVRKAFKSHVSTL  
 TDLOPYMROFVAHLOETSPLRDAVVIEOSSSLNEASSGLFDVFLRFMCHHAVRIRGK  
 SYVOCOGIPOGSILSTLLCSLCYGD MENKLFAGIRRDGILLRLVDDFLLVTPHLTHA  
 5 KTFLRTLVRGVPEYGCVVNL RKT VVNF PVEDEALGGTAFVOMPAHGLFPWCGLLLDT  
 RTLEVOSDYSSYARTSIRASLTENRGFKAGRNMRRKLEFVLRRLKCHSLFLDLVNLSL  
 OTVCTNIYKILLLOAYRFHACVLQLPFHOOVWKNPTFFLRVISDTASLCYSILKAKN  
 AGMSLGAKGAAGPLPSEAVOWLCHOAFLKLTRHRVTYVPLLGLSLRTAOTOLSRKLP  
 GTTLTALEAAAANPALPSDFKTILD [L] EOKLISEEDL [NSAVD] HHHHHH

10

### Expression of hTERT in Insect Cells

The following example details the construction of telomerase-expressing insect cell expression vectors to produce large quantities of full-length, biologically active hTERT (SEQ ID NO:2, SEQ ID NO:4).

#### *Baculovirus Expression Vector pBlueBacHis2 B and Full Length hTERT*

The telomerase coding sequence of interest was cloned into the baculovirus expression vector pVL1393 (Invitrogen, San Diego, CA). This construct was subsequently cotransfected into *Spodoptera frugiperda* (sf-9) cells with linearized DNA from *Autographa californica* nuclear polyhedrosis virus (Baculogold-AcMNPV). The recombinant baculoviruses obtained were subsequently plaque purified and expanded following published protocols.

This expression vector provides for expression in insect cells of high levels of full-length hTERT protein. Expression is driven by a baculoviral polyhedrin gene promoter. No exogenous codons were introduced by the cloning.

To produce large quantities of full-length, biologically active hTERT (SEQ ID NO:2), the baculovirus expression vector pBlueBacHis2 B (Invitrogen, San Diego, CA) was selected as a source of control elements. The hTERT-coding insert consisted of nucleotides 707 to 4776 of the hTERT insert in plasmid pGRN121 (SEQ ID NO:1), which includes the full-length sequence encoding hTERT (SEQ ID NO:2).

A full length hTERT with a His6 and Anti-Xpress tags (Invitrogen) was also constructed. This vector contains an insert consisting of nucleotides 707 to 4776 of the hTERT insert from the plasmid pGRN121 (SEQ ID NO:1). The vector directs expression in insect

cells of high levels of full length hTRT protein fused to a cleavable 6-histidine and Anti-Xpress tags (SEQ ID NO:33), and the amino acid sequence of the fusion protein is shown below; (-\*-) denotes enterokinase cleavage site:

5 MPRGSHHHHHHGMASMTGGQQMGRDLYDDDDL - \*-DPSSRSAAGTMEFA  
 AASTQRCVLLRTWEALAPATPAMPRAVRSLLRSHYREVLPLATFV  
 RRLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFRQVSCL  
 KELVARVLQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTV  
 TDALRGSGAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQVCPPPLY  
 10 QLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRG  
 GSASRSLPLPKRPRRGAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSP  
 ARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVY  
 AETKHFYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTP  
 RRLPRLPQRYWQMRPLFLELLGNHAQCPYGVLLKTHCPLRAAVTPAAGV  
 CAREKPGQSVAAPPEEDTDPRRLVQLLRQHSSPWQVYGFVRACLRLRVPP  
 15 GLWGSRHNERFLRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSP  
 GVGCVPAAEHRLREEILAKFLHWLMSVYVVELLRSEFFYVTETTFQKNRLF  
 FYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIP  
 KPDGLRPIVNMDYVVGARTFRREKRAERLTSRVKALFSVLNYERARRPGL  
 LGASVLGLDDIHRWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASI  
 20 IKPQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQETSPLRDA  
 VVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYVQCQGI PQGSILSTLLCSLCY  
 GDMENKLFAGIRRDGLLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLRKT  
 VVNFVPEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYARTSIRASLTF  
 NRGFKAGRNMRRKLFGLVRLKCHSLFLDLQVNSLQTVCTNIYKILLQAYRFHACVL  
 25 QLPFHQQVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLC  
 HQAFLKLTRHRVTYVPLLGSRLTAQTQLSRKLPGTTLTALEAAANPALPSDFKTIL  
 D

### 30 ***Baculovirus Expression Vector pBlueBac4.5 and Full Length hTRT Protein***

To produce large quantities of full-length, biologically active hTRT (SEQ ID NO:2), a second baculovirus expression vector, pBlueBac4.5 (Invitrogen, San Diego, CA) was constructed. The hTRT-coding insert consisted of nucleotides 707 to 4776 of the hTRT from the plasmid pGRN121 (SEQ ID NO:1). This nucleotide sequence includes the 3full-length sequence encoding hTRT (SEQ ID NO:2).

***Baculovirus Expression Vector pMelBacB and Full Length hTRT Protein***

To produce large quantities of full-length, biologically active hTRT (SEQ ID NO:2), a third baculovirus expression vector, pMelBacB (Invitrogen, San Diego, CA) was constructed. The hTRT-coding insert also consists of nucleotides 707 to 4776 of the hTRT insert from the plasmid pGRN121 (SEQ ID NO:1). This nucleotide sequence includes the full-length sequence encoding hTRT (SEQ ID NO:2).

pMelBacB directs expression of full length hTRT (SEQ ID NO:2) in insect cells to the extracellular medium through the secretory pathway using the melittin signal sequence. High levels full length hTRT (SEQ ID NO:2) are thus secreted. The melittin signal sequence is cleaved upon excretion, but is part of the protein pool that remains intracellularly. The sequence (SEQ ID NO:38) of the fusion protein encoded by the vector is shown below:

```

      (MKFLVNVALVFMVYIYIYA) - *-DPSSRSAAGTMEFAAASTQRCVLL
      RTWEALAPATPAMPRAVRSLLRSHYREVLPLATFVRRLGPOGW
15    RLVRGDPAAFRALVAQCLVCPWDARPPPAAPSFRQVSCLKELVARV
      LQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRG
      SGAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAA
      TQARPPPHASGPRRLGCERAWNHSVREAGVPLGLPAPGARRRGGSASR
      SLPLPKRPRRGAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAE
20    EATSLEGALSGTRHSHPSVGRQHAGPPSTSRPPRPWDTPCPPVYAETKH
      FLYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRL
      PQRYWQMRPLFLELLGNHAQCPYGVLLKTHCPLRAAVTPAAGVCAREKP
      QGSVAAPEEEDTDPRLVQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSR
      HNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSPGVGCVPA
25    AEHRLREEILAKFLHWLMSVYVVELLRSFFYVTETTFQKNRLFFYRKS VWS
      KLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRP IVN
      MDYVVGARTFRREKRAERLTSRVKALFVNLNERARRPGLLGASVLGLDD
      IHRAWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVR
      RYAVVQKAAHGHVRKAFKSHVSTLTDLPYMRQFVAHLQETSPLRDAVVIEQSSSLN
30    EASSGLFDVFLRFMCHHAVRIRGKSYVQCQGI PQGSILSTLLCSLCYGD MENKLFAG
      IRRDGLLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNL RKT VVNF PVEDEA
      LGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYARTSIRASLTFNRGFKAGRNM
      RRKLFGLRLKCHSLFLDLQVNSLQTVCTNIYKILLQAYRFHACVLQLP FHQ QVWK
      NPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTR
35    HRVTYVPLLGLSLRTAQTQLSRKLP GTTTLTALEAAANPALPSDFKTILD

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**Expression of hTRT in Mammalian Cells**

The recombinant hTERT of the invention can be produced in large quantities as full-length, biologically active hTERT (SEQ ID NO:2) in a variety of mammalian cell lines. This biologically active, mammalian produced hTERT is useful in many embodiments of the invention, as discussed above.

5 *hTERT Expressed in 293 Cells using Episomal Vector pEBVHis*

An episomal vector, pEBVHis (Invitrogen, San Diego, CA) was engineered to express an hTERT (SEQ ID NO:2) fusion protein comprising hTERT fused to an N-terminal extension epitope tag, the Xpress epitope (Invitrogen, San Diego, CA) (designated pGRN122). A control vector was also constructed containing as an insert the antisense sequence of hTERT and the epitope tag, coding sequence and so is useful as a negative control (the control plasmid designated pGRN123). The vector was transfected into 293 cells and translated hTERT identified and isolated using an antibody specific for the Xpress epitope. pEBVHis is a hygromycin resistant EBV episomal vector that expresses the protein of interest fused to an N-terminal peptide. Cells carrying the vector are selected and expanded, then nuclear and cytoplasmic extracts prepared. These and control extracts are immunoprecipitated with anti-Xpress antibody, and the immunoprecipitated beads are tested for telomerase activity by conventional assay.

The following list of expression plasmids is provided for illustrative purposes.

20

pGRN121

EcoRI fragment from lambda clone 25-1.1.6 containing the entire cDNA encoding hTERT protein inserted into the EcoRI site of pBluescriptIISK+ such that the 5' end of the cDNA is near the T7 promoter in the vector.

25

pGRN122

NotI fragment from pGRN121 containing the hTERT coding sequence inserted into the NotI site of pEBVHisA so that the coding sequence is operably linked to the RSV promoter. This plasmid expresses a fusion protein composed of a His6 flag fused to the N-terminus of the hTERT protein.

#### pGRN124

pGRN121 was deleted of all APA1 sites followed by deletion of the MSC1-HINC2 fragment containing the 3'UTR ("untranslated region"). The Nco-XbaI fragment containing the stop codon of the hTERT coding sequence was then inserted into the Nco-XbaI sites of pGRN121 to make a plasmid equivalent to pGRN121 except lacking the 3'UTR, which may be preferred for increased expression levels in some cells.

#### pGRN125

NotI fragment from pGRN124 containing the hTERT coding sequence inserted into the NotI site of pBBS235 so that the open reading frame is in the opposite orientation of the Lac promoter.

#### pGRN126

NotI fragment from pGRN124 containing the hTERT coding sequence inserted into the NotI site of pBBS235 so that the hTERT coding sequence inserted is in the same orientation as the Lac promoter.

#### pGRN127

The oligonucleotide 5'-

20GCGCACGTGGGAAGCCCTGGCagatctgAattCCaCcATGCCGCGCGCTCCCCGCTG-3' was used in vitro mutagenesis of pGRN125 to convert the initiating ATG codon of the hTERT coding sequence into a Kozak consensus sequence and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR (ampicillin resistant) and COD1941 was used to convert CatR (chloramphenicol resistant) to CatS (chloramphenicol sensitive).

#### pGRN130

The oligonucleotide 5'-

CGGGACGGGCTGCTCCTGCGTTTGGTGGAcGcgTTCTTGTTGGTGACACCTCACCT 30ACC-3' was used in in Vitro mutagenesis to convert the Asp869 codon into an Ala codon

(i.e. the second Asp of the DD motif was converted to an Alanine to make a dominant/negative variant protein). This also created an MluI site. Also, the oligonucleotide 5'-TGCGCACGTGGGAAGCCCTGGCagatctgAattCCaCcATGCCGCGCGCTCCCCGCTG-3' was used in in Vitro mutagenesis to convert the initiating ATG codon of the hTRT coding sequence into a Kozak consensus sequence and create EcoRI and BglII sites for cloning. Also, COD2866 was used to convert AmpS to AmpR (ampicillin resistant) and COD1941 was used to convert CatR (chloramphenicol resistant) to CatS (chloramphenicol sensitive).

#### pGRN133

1 HcoRI fragment from pGRN121 containing the hTRT coding sequence inserted into the EcoRI site of pBBS212 so that the hTRT protein is expressed under the control of the MPSV promoter.

#### pGRN134

1 HcoRI fragment from pGRN121 containing the hTRT coding sequence inserted into the EcoRI site of pBBS212 so that the antisense of the hTRT coding sequence is expressed under the control of the MPSV promoter.

#### pGRN135

20 pGRN126 was digested to completion with MscI and SmaI and religated to delete over 95% of the hTRT coding sequence inserted. One SmaI-MscI fragment was re-inserted during the process to recreate the Cat activity for selection. This unpurified plasmid was then redigested with SalI and EcoRI and the fragment containing the initiating codon of the hTRT coding sequence was inserted into the SalI-EcoRI sites of pBBS212. This makes an antisense  
25 expression plasmid expressing the antisense of the 5'UTR and 73 bases of the coding sequence.

#### pGRN136

HindIII-SalI fragment from pGRN126 containing the hTRT coding sequence inserted into the 3 HindIII-SalI sites of pBBS242.

pGRN137

SalI-Sse8387I fragment from pGRN130 containing the Kozak sequence inserted into the SalI-Sse8387I sites of pGRN136.

pGRN139

The oligonucleotide

CTGCCCTCAGACTTCAAGACCATCCTGGACTACAAGGACGACGATGACAAATGA  
ATTCAGATCTGCGGCCGCCACCGCGGTGGAGCTCCAGC was used to insert the IBI  
Flag at the C-terminus of

hTERT in pGRN125 and create ECOR1 and BGL2 sites for cloning.

pGRN145

EcoRI fragment from pGRN137 containing the hTERT coding sequence inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of hTERT mRNA. The hTERT coding sequence is oriented so that it is expressed under the control of the MPSV promoter.

pGRN146

Sse8387I-NotI fragment from pGRN130 containing the D869A mutation of hTERT inserted into the Sse8387I-NotI sites of pGRN137.

pGRN147

Sse8387I-NotI fragment from pGRN139 containing the IBI Flag inserted into the Sse8387I-NotI sites of pGRN137.

25

pGRN151

EcoRI fragment from pGRN147 containing the hTERT coding sequence inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of the hTERT mRNA. The hTERT coding sequence is oriented so that it is expressed under the control of the MPSV promoter.

## pGRN152

EcoRI fragment from pGRN146 containing the hTRT coding sequence inserted into the EcoRI site of pBBS212 to remove the portion of the sequence corresponding to the 5'UTR of the hTRT. The hTRT coding sequence is oriented so that it is expressed under the control of the MPSV promoter.

## pGRN154

Eam1105I fragment from pGRN146 containing the Kozak consensus sequence and the 5' end of the hTRT coding sequence inserted into the Eam1105I sites of pGRN147 to make an hTSP expression plasmid that expresses an hTRT variant protein with a Kozak consensus sequence, and a protein having the D869->A mutation, fused to the IBI flag protein.

## EXAMPLE 7

CO-EXPRESSION OF hTRT AND hTR IN VITRO

15 In this example, the coexpression of hTRT and hTR using an *in vitro* cell-free expression system is described. These results demonstrate that the hTRT peptide encoded by pGRN121 encodes a catalytically active telomerase proteins and that reconstitution of the telomerase RNP can be accomplished *in vitro* using recombinantly expressed hTRT and hTR.

Telomerase activity was reconstituted by adding linearized plasmids of hTRT (pGRN 121; 1ug DNA digested with Xba I) and hTR (phTR+1; 1 ug digested with Fsp I) to a coupled transcription-translation reticulocyte lysate system (Promega TNT<sup>TM</sup>). phTR+1 is a plasmid which when linearized with Fsp I, will generate a 445 nt transcript beginning with nucleotide +1 and extending to nucleotide 445. (Autexier et al., 1996, *EMBO J* 15:5928). For a 50 ul reaction the following components were added: 2 ul TNT<sup>TM</sup> buffer, 1 ul TNT<sup>TM</sup> RNA polymerase, 1 ul 1 mM amino acid mixture, 40 units Rnasin<sup>TM</sup> RNase inhibitor, 1 ug each, linearized template DNA, and 25 ul TNT\* reticulocyte lysate. Components were added in the ratio recommended by the manufacturer and were incubated for 90 min at 30 oC. Transcription was under the direction of the T7 promoter and could also be carried out prior to the addition reticulocyte lysate with similar results. 5 and 10 ul of the programmed transcription-translation was assayed for telomerase activity as previously described



(Autexier et al., supra) using 20 cycles of PCR to amplify the signal.

The results of the reconstitution are shown in **Figure 10**. For each transcription/translation reaction there are 3 lanes: The first 2 lanes are duplicate assays and the third lane is a heat denatured (95°C, 5 min) sample to rule out PCR generated artifacts.

5 As shown in **Figure 10**, reticulocyte lysate alone has no detectable telomerase activity (lane 6). Similarly, no detectable activity is observed when either hTR alone (lane 1) or full length hTERT gene (lane 4) are added to the lysate. When both components are added (lane 2), telomerase activity is generated as demonstrated by the characteristic repeat ladder pattern. When the carboxy-terminal region of the hTERT gene is removed by digestion of the vector with *NcoI* ("truncated hTERT") telomerase activity is abolished (lane 3). Lane 5 shows that translation of the truncated hTERT also not generate telomerase activity. Lane "R8" shows a positive control (TSR8 quantitation standard (5'-ATTCCGTCGAGCAGAGTTAG[GGTTAG]7-3')).

15

## EXAMPLE 8

### PRODUCTION OF ANTI-HTRT ANTIBODIES

#### A) Production of anti-hTERT antibodies against hTERT peptides

To produce anti-hTERT antibodies the following peptides from hTERT were synthesized with the addition of C (cysteine) as the amino terminal residue.

20 S-1: FFY VTE TTF QKN RLF FYR KSV WSK

S-2: RQH LKR VQL RDV SEA EVR QHR EA

S-3: ART FRR EKR AER LTS RVK ALF SVL NYE

A-3: PAL LTS RLR FIP KPD GLR PIV NMD YVV

The cysteine moiety used to immobilize the peptides to BSA and KLH [keyhole limpet hemocyanin] carrier proteins. The KLH-peptides were used as antigen. The BSA-peptides conjugates served as material for ELISAs for testing the specificity of immune antisera.

The KLH-peptide conjugates were injected into New Zealand White rabbits. The initial injections are made by placing the injectant proximal to the axillary and inguinal lymph nodes. Subsequent injections were made intramuscularly. For initial injections, the antigen was emulsified with Freund's complete adjuvant; for subsequent injections, Freund's

incomplete adjuvant was used. Rabbits follow a three week boost cycle, in which 50 ml of blood yielding 20-25 ml of serum is taken 10 days after each boost. Antisera against each of the four peptides recognized the hTRT moiety of recombinant hTRT fusion protein (GST-HIS<sub>8</sub>-hTRT-fragment #3; see **Example 6**) on western blots.

5           Using a partially purified telomerase fraction from human 293 cells (approximately 1000-fold purification compared to a crude nuclear extract) was produced as described in co-pending U.S. patent application Serial No. 08/833,37 (see also, PCT application No. 97/06012) and affinity purified anti-S-2 antibodies, a 130 kd protein doublet could be detected on a western blot. A sensitive chemiluminescence detection method was employed (SuperSignal chemiluminescence substrates, Pierce) but the signal on the blot was weak, suggesting that hTRT is present in low or very low abundance in these immortal cells. The observation of a doublet is consistent with a post-translational modification of hTRT, i.e., phosphorylation or glycosylation.

For affinity purification, the S-2 peptide was immobilized to SulfoLink (Pierce, Rockford IL) through its N-terminal Cysteine residue according to the manufacturer's protocol. First bleed serum from a rabbit immunized with the KLH-S-2 peptide antigen was loaded over the S-2-SulfoLink and antibodies specifically recognizing the S-2 peptide were eluted.

## 20       B) Production of anti-hTRT antibodies against hTRT fusion proteins

GST-hTRT fusion proteins were expressed in E. coli from the GST-hTRT fragment #4 and the GST-HIS<sub>8</sub>-hTRT fragment #3 vectors described in **Example 6**. The fusion proteins were purified as insoluble protein, and the purity of the antigens was assayed by SDS polyacrylamide gels and estimated to be about 50% pure for GST-hTRT fragment #4 recombinant protein and more than 90% pure for GST-HIS<sub>8</sub>-hTRT fragment #3 recombinant protein. These recombinant proteins were used to immunize both New Zealand White rabbits and female Balb/c mice. For initial injections, the antigen was emulsified with Freund's complete adjuvant; for subsequent injections, Freund's incomplete adjuvant is used. Rabbits and mice follow a three week boost cycle, in which blood is taken 10 days after each boost.

30           The first bleeds from both the mice and rabbits were tested for the presence of

anti-hTERT antibodies after removal of anti-GST antibodies using a matrix containing immobilized GST. The antisera were tested for anti-hTERT antibodies presence by western blotting, using immobilized recombinant GST-hTERT fusion protein, and by immunoprecipitation using partially purified native telomerase enzyme. No signal was observed in these early bleeds; titers of anti-hTERT antibodies are expected to increase in subsequent bleeds.

#### EXAMPLE 9

##### DETECTION OF A hTERT mRNA CORRESPONDING TO $\Delta$ 182 RNA VARIANT

10 Poly A<sup>+</sup> RNA from human testis and the 293 cell lines was reverse transcribed using RT-PCR and nested primers. The first primer set was TCP1.1 and TCP1.15; the second primer set was TCP1.14 and billTCP6. Amplification from each gave two products differing by 182 bp; the larger and smaller products from testis RNA were sequenced and found to correspond exactly to pGRN121 (SEQ ID NO \_\_) and the 712562 clone (SEQ ID NO \_\_), respectively. The variant hTERT RNA product has been observed in mRNA from SW39i, OVCAR4, 293, Testes, BJ and IMR90 cells.

Additional experiments were carried out to demonstrate that the  $\Delta$ 182 cDNA was not an artifact of reverse transcription. Briefly, full-length hTERT RNA (i.e., without the deletion) was produced by *in vitro* transcription of pGRN121 for use as a template for RT-PCR. Separate cDNA synthesis reactions were carried out using Superscript® reverse transcriptase (Bethesda Research Laboratories, Bethesda MD) at 42° or 50°C, and with random-primers or a specific primer. After 15 PCR cycles the longer product was detectable; however, the smaller product (i.e., corresponding to the deletion) was not detectable even after 30 or more cycles. This indicates that the RT-PCR product is not artifactual.

25

#### EXAMPLE 10

##### SEQUENCING OF TESTIS hTERT mRNA

The sequence of the testes form of hTERT RNA was determined by direct manual sequencing of DNA fragments generated by PCR from Testes cDNA (Marathon Testes cDNA, Clontech, San Diego CA) using a Therm(o-S)equenase radiolabeled terminator

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cycle sequencing kit (Amersham Life Science). The PCR reactions were performed by nested PCR, as shown in **Table 5**, except where noted. In all cases a negative control reaction with primers but no cDNA was performed. The absence of product in the control reaction demonstrated that the products derived from the reaction with cDNA present were not due to contamination of hTRT from pGRN121 or other cell sources (e.g., 293 cells). The DNA fragments were excised from agarose gels to purify the DNA prior to sequencing.

The testis mRNA sequence corresponding to bases 27 to 3553 of the pGRN121 insert sequence (**SEQ. ID. NO: 1**), and containing the entire hTRT ORF (bases 56 to 3451) was obtained. There were no differences between the testes and the pGRN121 sequences from in this region.

TABLE 5

Fragment	primer set 1	primer set 2	final size	primers for seq
0A	na	K320 / K322	208	K320,K322
A	K320 / TCP1.43	TCP1.40 / TCP1.34	556	TCP1.52,TCP1.39,K322,TCP1.40,TCP1.41,TCP1.30,TCP1.34,TCP1.49
B	TCP1.42 / TCP1.32B	TCP1.35 / TCP1.21	492	TCP1.35,TCP1.28,TCP1.38,TCP1.21,TCP1.46,TCP1.33,TCP1.48
C	TCP1.65 / TCP1.66	TCP1.67 / TCP1.68	818	TCP1.67,TCP1.32,TCP1.69,TCP1.68,TCP1.24,TCP1.44,K303
D2	K304 / billTCP6	LT1 / TCP1.6	546	LT2,LT1,TCP1.6,bTCP4,TCP1.13,TCP1.77,TCP1.1
D3	TCP1.12 / TCP1.7	TCP1.14 / TCP1.15	604	TCP1.6,TCP1.14,TCP1.73,TCP1.78,TCP1.25,TCP1.15,TCP1.76
EF	na	TCP1.74 / TCP1.7	201	TCP1.74,TCP1.7,TCP1.75,TCP1.15,TCP1.3
E	TCP1.3 / TCP1.4	TCP1.2 / TCP1.9	687	TCP1.2,TCP1.8,TCP1.9,TCP1.26
F	TCP1.26 / UTR2	TCP1.10 / TCP1.4	377	TCP1.4,TCP1.10,TCP1.11

## EXAMPLE 11

## DETECTION OF HTRT MRNA BY RNASE PROTECTION

10 RNase protection assays can be used to detect, monitor, or diagnose the presence of an hTERT mRNA or variant mRNA. One illustrative RNase protection probe is an *in vitro* synthesized RNA comprised of sequences complementary to hTERT mRNA sequences and additional, non-complementary sequences. The latter sequences are included to distinguish the full-length probe from the fragment of the probe that results from a positive result in the assay: in a positive assay, the complementary sequences of the probe are protected from RNase digestion, because they are hybridized to hTERT mRNA. The non-complementary sequences are digested away from the probe in the presence of RNase and target complementary nucleic acid.

Two RNase protection probes are described for illustrative purposes; either can be used in the assay. The probes differ in their sequences complementary to hTERT, but contain identical non-complementary sequences, in this embodiment, derived from the SV40 late mRNA leader sequence. From 5'-3', one probe is comprised of 33 nucleotides of non-complementary sequence and 194 nucleotides of sequence complementary to hTERT nucleotides 2513 - 2707 for a full length probe size of 227 nucleotides. From 5'-3', the second probe is comprised of 33 nucleotides of non-complementary sequence and 198 nucleotides of sequence complementary to hTERT nucleotides 2837 - 3035 for a full length probe size of 231 nucleotides. To conduct the assay, either probe can be hybridized to RNA, i.e., polyA<sup>+</sup> RNA, from a test sample, and T1 ribonuclease and RNase A are then added. After digestion, probe RNA is purified and analyzed by gel electrophoresis. Detection of a 194 nucleotide fragment of the 227 nucleotide probe or a 198 nucleotide fragment of the 231 nucleotide probe is indicative of hTERT mRNA in the sample.

25 The illustrative RNase protection probes described in this example can be generated by *in vitro* transcription using T7 RNA polymerase. Radioactive or otherwise labeled ribonucleotides can be included for synthesis of labeled probes. The templates for the *in vitro* transcription reaction to produce the RNA probes are PCR products. These illustrative probes can be synthesized using T7 polymerase following PCR amplification of 30 GRN121 DNA using primers that span the corresponding complementary region of the

hTRT gene or mRNA. In addition, the downstream primer contains T7 RNA polymerase promoter sequences and the non-complementary sequences.

For generation of the first RNase protection probe, the PCR product from the following primer pair (T701 and reverse01) is used:

T701 5'-GGGAGATCT TAATACGACTCACTATAG ATTCA GGCCATGGTG CTGCGCCGGC TGTCA GGCTCCC ACGACGTAGT CCATGTTTAC-3' [SEQ ID NO: \_\_\_\_]; and reverse01 5'-GGGTCTAGAT CCGGAAGAGTGT CTGGAGCAAG-3' [SEQ ID NO: \_\_\_\_].

10 For generation of the second RNase protection probe, the PCR product from the following primer pair (T702 and reverse02) is used:

T702 5'-GGGAGATCT TAATACGACTCACTATAG ATTCA GGCCATGGTG CTGCGCCGGC TGTCA GGGCG GCCTTCTGGA CCACGGCATA CC-3' [SEQ ID NO: \_\_\_\_]; and reverse02 5'-G GTCTAGA CGATATCC ACAGGGCCTG GCGC-3' [SEQ ID NO: \_\_\_\_].

## EXAMPLE 12

### CONSTRUCTION OF A PHYLOGENETIC TREE COMPARING HTRT AND OTHER REVERSE TRANSCRIPTASES

20 A phylogenetic tree (**Figure 6**) was constructed by comparison of the seven RT domains defined by Xiong and Eickbush (1990, *EMBO J.* 9:3353). After sequence alignment of motifs 1, 2, and A-E from 4 TRTs, 67 RTs, and 3 RNA polymerases, the tree was constructed using the NJ (Neighbor Joining) method (Saitou and Nei, 1987, *Mol. Biol. Evol.* 4:406). Elements from the same class that are located on the same branch of the tree  
25 are simplified as a box. The length of each box corresponds to the most divergent element within that box.

The TRTs appear to be more closely related to RTs associated with msDNA, group II introns, and non-LTR (Long Terminal Repeat) retrotransposons than to the LTR-retrotransposon and viral RTs. The relationship of the telomerase RTs to the non-LTR  
30 branch of retroelements is intriguing, given that these latter elements have replaced

telomerase for telomere maintenance in *Drosophila*. However, the most striking finding is that the TRTs form a discrete subgroup, almost as closely related to the RNA-dependent RNA polymerases of plus-stranded RNA viruses such as poliovirus as to any of the previously known RTs. Considering that the four telomerase genes come from evolutionarily distant organisms -- protozoan, fungi, and mammal -- this separate grouping cannot be explained by lack of phylogenetic diversity in the data set. Instead, this deep bifurcation suggests that the telomerase RTs are an ancient group, perhaps originating with the first eukaryote.

GenBank protein identification or accession numbers used in the phylogenetic analysis were: msDNAs (94535, 134069, 134074, 134075, 134078), group II introns (483039, 101880, 1332208, 1334433, 1334435, 133345, 1353081), mitochondrial plasmid/RTL (903835, 134084), non-LTR retrotransposons (140023, 84806, 103221, 103353, 134083, 435415, 103015, 1335673, 85020, 141475, 106903, 130402, U0551, 903695, 940390, 2055276, L08889), LTR retrotransposons (74599, 85105, 130582, 99712, 83589, 84126, 1479443, 224319, 130398, 130583, 1335652, 173088, 226407, 101042, 1078824), hepadnaviruses (18876, 1706510, 118894), caulimoviruses (331554, 130600, 130593, 93553), retroviruses (130601, 325465, 74601, 130587, 130671, 130607, 130629, 130589, 130631, 1346746, 130651, 130635, 1780973, 130646). Alignment was analyzed using ClustalW 1.5 [J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* 22, 20673 (1994)] and PHYLIP 3.5 [J. Felsenstein, *Cladistics* 5, 164 (1989)].

### EXAMPLE 13

#### **Transfection of cultured human fibroblasts (BJ) with control plasmid and plasmid encoding hTRT**

25            This example demonstrates that expression of recombinant hTRT protein in a mammalian cell results in the generation of an active telomerase.

Subconfluent BJ fibroblasts were trypsinized and resuspended in fresh medium (DMEM/199 containing 10% Fetal Calf Serum) at a concentration of  $4 \times 10^6$  cells/ml. The cells were transfected using electroporation with the BioRad Gene Pulser™  
30 electroporator. For electroporation, 500  $\mu$ l of the cell suspension were placed in an



electroporation cuvette (BioRad, 0.4 cm electrode gap). Plasmid DNA (2 µg) was added to the cuvettes and the suspension was gently mixed and incubated on ice for 5 minutes. The control plasmid (pBBS212) contained no insert behind the MPSV promoter and the experimental plasmid (pGRN133) expressed hTERT from the MPSV promoter. The cells were electroporated at 300 Volts and 960 µFD. After the pulse was delivered, the cuvettes were placed on ice for approximately 5 minutes prior to plating on 100 mm tissue culture dishes in medium. After 6 hours, the medium was replaced with fresh medium. 72 hours after the transfection, the cells were trypsinized, washed once with PBS, pelleted and stored frozen at -80°C. Cell extracts were prepared at a concentration of 25,000 cells/µl by a modified detergent lysis method (see Bodnar et al., 1996, *Exp. Cell Res.* 228:58; Kim et al., 1994, *Science* 266:2011, and as described patents and publications relating to the TRAP assay, *supra*) and telomerase activity in the cell extracts was determined using a modified PCR-based TRAP assay (Kim et al 1994, Bodnar et al 1996). Briefly, 5 x10<sup>4</sup> cell equivalents were used in the telomerase extension portion of the reaction. This reaction mixture was then extracted once with phenol/chloroform and once with chloroform and one-fifth of the extracted material was used in the PCR amplification portion of the TRAP reaction (approximately 10,000 cell equivalents). One half of the TRAP reaction was loaded onto the gel for analysis, such that each lane in **Figure 25** represents reaction products from 5,000 cell equivalents.

20

#### **EXAMPLE 14**

##### **PROMOTER REPORTER CONSTRUCT**

This example describes the construction of plasmid in which a reporter gene is operably linked to the hTERT upstream sequence containing promoter elements. The vectors have numerous uses, including identification of *cis* and *trans* transcriptional regulatory factors *in vivo* and for screening of agents capable of modulating (e.g., activating or inhibiting) hTERT expression (e.g., drug screening). Although a number of reporters may be used (e.g., Firefly luciferase, β-glucuronidase, β-galactosidase, chloramphenicol acetyl transferase, and GFP), the human secreted alkaline phosphatase (SEAP; Clontech) was used for initial experiments. The SEAP reporter gene encodes a truncated form of the

placental enzyme which lacks the membrane anchoring domain, thereby allowing the protein to be efficiently secreted from transfected cells. Levels of SEAP activity detected in the culture medium have been shown to be directly proportional to changes in intracellular concentrations of SEAP mRNA and protein (Berger et al., 1988, *Gene* 66:1; Cullen et al., 1992, *Meth. Enzymol.* 216:362).

Four constructs (pGRN 148, pGRN 150, pSEAP2 basic (no promoter sequences = negative control) and pSEAP2 control (contains the SV40 early promoter and enhancer) were transfected in duplicate in mortal and immortal cells.

Plasmid pGRN148 was constructed as illustrated in **Figure 9**. Briefly, a Bgl2-1Bco47III fragment from pGRN144 was digested into the Bgl2 NruI site of pSeap2Basic. [??] A second reporter-promoter, Plasmid pGRN150, includes sequences from the hTERT intron described in **Example 3**, to employ regulatory sequences that may be present in the intron. The initiating Met is mutated to Leu, so that the second ATG following the promoter region will be the initiating ATG of the SEAP ORF).

15 Stable transformants of pGRN148 are made in telomerase negative and telomerase positive cells by cotransformation with eukaryotic selectable marker (such as *neo*) according to Ausubel et al., 1997, *supra*. The resulting cell lines are used for screening of putative telomerase modulatory agents.

20

## EXAMPLE 15

### SUBCELLULAR LOCALIZATION OF HTRT

A fusion protein having hTERT and enhanced green fluorescent protein (EGFP; Cormack et al., 1996, *Gene* 173:33) regions was constructed as described below. The EGFP moiety provides a detectable tag or signal so that the presence or location of the fusion protein can be easily determined. Because EGFP-fusion proteins localize in the correct cellular compartments, this construct may be used to determine the subcellular location of hTERT protein.

3A. Construction of pGRN 138.

A vector for expression of an hTERT-EGFP fusion protein in mammalian cells was constructed by placing the EcoRI insert from **pGRN124** (see **Example 6**) into the EcoRI site of pEGFP-C2 (Clontech, San Diego, CA). The amino acid sequence of the fusion protein is provided below (SEQ. ID. NO. \_\_\_\_). EGFP residues are in bold, residues encoded by the 5' untranslated region of hTERT mRNA are underlined, and the hTERT protein sequence is in normal font.

MVSKGEELFTGVVPIILVELDGDVNGHKFSVS GEGEGDATYGKLTTLKFICTTGKLPVPWPPT  
 LVTTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTL  
 VNRIELKGIDFKEDGNILGHKLEYNYSNHNVIIMADKQKNGIKVNFKIRHNIEDGSVQLA  
 10HYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDMVLLLEFVTAAGITLGMDELYKS  
 GRTQISSSSFEFAAASTORCVLLRTWEALAPATPAMPAPRCRAVRSLLRSHYREVLPLA  
 TFVRRLLGPQGWRLVQRGDPAAFRALVAQCLVCVPWDARPPPAAPSFRQVSCLKELVARVL  
 QRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVG  
 DDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNH  
 15VREAGVPLGLPAPGARRRGGSASRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGP  
 SDRGFCVVSPPARPAEEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVY  
 AETKHFYSSGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQR  
 YWQMRPLFLELLGNHAQCYPYGVLLKTHCPLRAAVTPAAGVCAREKPPQGSVAAPPEEDTDP  
 RRLVQLLRQHSSPWQVYGFVRACLRRLVPPGLWGSRHNRRLRNTKKFISLGKHAKLSL  
 20ELTWKMSVRDCAWLRRSPGVGCVPAAEHRLREEILAKFLHWMMSVYVVELLRSSFYVTE  
 TTFQKNRLFFYRPSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIP  
 KPDGLRPIVNM DYVVGARTFRREKRAERLTSRVKALFVSLNYERARRPGLLGASVLGLDD  
 IHRAWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYA  
 VVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIEQSSSLNEASSGL  
 25DVFLRFMCHHAVRIRGKSYVQCQGI PQGSILSTLLCSLCYGD MENKLFAGIRRDGLLLR  
 LVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLKTVVNFVVEDEALGGTAFVQMPAH  
 GLFPWCGLLLDTRTLEVQSDYSSYARTSIRASVTFNRGFKAGRNMRRKLFGLVRLKCHSL  
 FLDLQVNSLQTVCTNIYKILLLQAYRFHACVLQLPFHQVWKNPTFFLRVISDTASLCYS  
 ILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGLSLRTAQTQLSR  
 30LPGTTLTALEAAANPALPSDFKTILD

Other EGFP fusion constructs are made using partial (e.g., truncated) hTERT coding sequence and used, as described *infra*, to identify activities of particular regions of the hTERT polypeptide.

35

#### B. Uses of pGRN 138

Fluorescence microscopy studies of MDA breast cancer cells transfected with pGRN 138, are carried out to determine whether, as expected, transfection confers fluorescence to the nucleus of the cell while transfection of a vector encoding EGFP alone

confers fluorescence to the cytoplasm and not the nucleus.

The fusion construct described in this example, or a construct of EGFP and a truncated form of hTERT, is used to assess the ability of hTERT and telomerase variants to enter a cell nucleus and localize at the chromosome ends. In addition, cells are stably or transiently transfected with pGRN138 are used for screening putative telomerase modulatory drugs or compounds. Agents that interfere with nuclear localization or telomere localization are identified as telomerase inhibitors.

In addition, FACS or other fluorescence-based methods are used to select cells expressing hTERT to provide homogeneous populations for drug screening, particularly when transient transfection of cells is employed.

#### **EXAMPLE 16**

##### **MUTATION OF hTERT FFYxTE MOTIF**

A vector encoding an hTERT mutant protein, "F560A," in which amino acid 560 of SEQ. ID. NO. 2 was changed from phenylalanine (F) to alanine (A) by site directed mutagenesis of pGRN121 using standard techniques. This mutation disrupts the TRT FFYxTE motif. The resulting F560A mutant polynucleotide was shown to direct synthesis of a full length hTERT protein as assessed using a cell-free reticulocyte lysate transcription/translation system in the presence of <sup>35</sup>S-methionine.

When the mutant polypeptide is co-translated with hTR, as described in Example 7, no telomerase activity was detected as observed by TRAP using 20 cycles of PCR, while a control hTERT/hTR did reconstitute activity. Using 30 cycles of PCR in the TRAP assay, telomerase activity was observable with the mutant hTERT, but was considerably lower than the control (wild-type) hTERT. These results indicate that this mutation has an effect on catalytic activity that is critical for optimal activity but which does not absolutely required for catalytic activity.

\*\*\*

The following clones described in the Examples have been deposited with the American Type Culture Collection, Rockville, MD 20852, USA:

Lambda phage  $\lambda$  25-1.1 ATCC accession number 209024

pGRN 121 ATCC accession number 209016

\*\*\*

5           The present invention provides novel methods and materials for diagnosis and treatment of telomerase-related diseases. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but  
10 instead should be determined with reference to the appended claims along with their full scope of equivalents.

          All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

15

\*\*\*

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scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

5

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WHAT IS CLAIMED IS:

1. An isolated, substantially pure, or recombinant protein preparation of a telomerase reverse transcriptase protein, said protein characterized by having an amino acid sequence:

Trp-R<sub>1</sub>-X<sub>7</sub>-R<sub>1</sub>-R<sub>1</sub>-R<sub>2</sub>-X-Phe-Phe-Tyr-X-Thr-Glu-X<sub>8,9</sub>-R<sub>3</sub>-R<sub>3</sub>-Arg-R<sub>4</sub>-X<sub>2</sub>-Trp

where X is any amino acid and a subscript refers to the number of consecutive residues, R<sub>1</sub> is leucine or isoleucine, R<sub>2</sub> is glutamine or arginine, R<sub>3</sub> is phenylalanine or tyrosine, and R<sub>4</sub> is lysine or histidine.

2. An isolated, substantially pure or recombinant nucleic acid that encodes a telomerase reverse transcriptase protein, said protein characterized by having an amino acid sequence:

Trp-R<sub>1</sub>-X<sub>7</sub>-R<sub>1</sub>-R<sub>1</sub>-R<sub>2</sub>-X-Phe-Phe-Tyr-X-Thr-Glu-X<sub>8,9</sub>-R<sub>3</sub>-R<sub>3</sub>-Arg-R<sub>4</sub>-X<sub>2</sub>-Trp

where X is any amino acid and a subscript refers to the number of consecutive residues, R<sub>1</sub> is leucine or isoleucine, R<sub>2</sub> is glutamine or arginine, R<sub>3</sub> is phenylalanine or tyrosine, and R<sub>4</sub> is lysine or histidine.

3. The protein preparation of claim 1 that is a recombinant telomerase reverse transcriptase protein having an amino acid sequence identical to the amino acid sequence of a naturally occurring telomerase reverse transcriptase protein selected from the group of organisms consisting of *Tetrahymena*, *Euploites*, *S. pombe*, and humans.

4. The nucleic acid of claim 2 that is a recombinant nucleic acid having a nucleotide sequence that encodes a recombinant protein of claim 3.

5. The nucleic acid of claim 4 that encodes hTERT.

6. A method of detecting a human telomerase reverse transcriptase (hTERT) gene product in a biological sample comprising:

a) contacting the biological sample with a probe that specifically binds the gene product, wherein the probe and the gene product form a complex, and detecting the complex; or,

b) specifically amplifying the gene product in the biological sample, wherein said gene product is a nucleic acid, and detecting the amplification product;

wherein the presence of the complex or amplification product is correlated with the presence of the hTERT gene product in the biological sample.

10

7. The method of claim 6, wherein the gene product is an RNA.

8. The method of claim 7, wherein the probe is a nucleic acid.

15 9. The method of claim 6, wherein the gene product is a polypeptide.

10. The method of claim 9, wherein the probe is an antibody.

11. The method of claim 6, wherein the biological sample is from a patient.

20

12. The method of claim 6, wherein the biological sample comprises at least one cell from an *in vitro* cell culture.

13. The method of claim 12, wherein the cell is human.

25

14. A method of detecting the presence of at least one immortal human cell in a biological sample comprising human cells, said method comprising the steps:

a) obtaining the biological sample comprising human cells; and,

b) detecting the presence in the sample of a cell having a high level of an hTERT gene product,



wherein the presence of a cell having a high level of the hTERT gene product is correlated with the presence of immortal cells in the biological sample.

15. A method for diagnosing a telomerase-related condition in a patient, comprising:
- a) obtaining a cell or tissue sample from the patient;
  - b) determining the amount of a human telomerase reverse transcriptase (hTERT) gene product in the cell or tissue; and,
  - c) comparing the amount of hTERT gene product in the cell or tissue with the amount in a healthy cell or tissue of the same type;

wherein a different amount of hTERT gene product in the sample from the patient and the healthy cell or tissue is diagnostic of a telomerase-related condition.

16. The method of claim 15 wherein the telomerase-related condition is cancer.

17. A method of diagnosing cancer in a patient, said method comprising:
- a) obtaining a biological sample from the patient; and,
  - b) detecting a human telomerase reverse transcriptase (hTERT) gene product in the patient sample;
- wherein the detection of the hTERT gene product in the sample is correlated with a diagnosis of cancer.

18. A method of diagnosing cancer in a patient, said method comprising:
- a) obtaining a patient sample;
  - b) determining the amount human telomerase reverse transcriptase (hTERT) gene product in the patient sample; and,
  - c) comparing the amount of hTERT gene product with a normal value for the sample,
- wherein an amount of the hTERT gene product in the patient that is greater than the normal value is diagnostic of cancer.

19. A method of diagnosing cancer in a patient, said method comprising:
- a) obtaining a patient sample containing at least one cell;
  - b) determining the amount of a hTRT gene product in a cell in the sample;

and,

- 5 c) comparing the amount of hTRT gene product in the cell with a normal value for the cell,

wherein an amount of the hTRT gene product greater than the normal value is diagnostic of cancer.

- 10 20. The method of claim 19, wherein the sample is believed to contain at least one malignant cell.

21. A method of providing a prognosis for a cancer patient, said method comprising:

- 15 a) determining the amount of hTRT gene product in a cancer cell obtained from the patient; and,

b) comparing the amount of hTRT in the cancer cell with a prognostic value of hTRT per cancer cell consistent with a prognosis for the cancer;

whereby an amount of hTR per cell in the sample that is at the prognostic value provides the particular prognosis.

22. A method for monitoring the ability of an anticancer treatment to reduce the proliferative capacity of cancer cells in a patient, said method comprising:

- a) making a first measurement of the amount of an hTRT gene product in at least one cancer cell from the patient;

b) making a second measurement of the level of the hTRT gene product in at least one cancer cell from the patient, wherein the anticancer treatment is administered to the patient before or at the same time as the second measurement; and,

- c) comparing the first and second measurements,
- 30 wherein a lower level of the hTRT gene product in the second measurement is

correlated with the ability of an anticancer treatment to reduce the proliferative capacity of cancer cells in the patient.

23. A kit for the detection of a hTERT gene or gene product, said kit comprising a container containing a molecule selected from an hTERT nucleic acid or subsequence thereof, an hTERT polypeptide or subsequence thereof, and an anti-hTERT antibody.

24. A method for increasing the proliferative capacity of a vertebrate cell, said method comprising:

introducing a recombinant polynucleotide into the cell,  
wherein said polynucleotide comprises a sequence encoding a human telomerase reverse transcriptase (hTERT) polypeptide,  
and wherein said sequence is operably linked to a promoter.

15

25. The method of claim 24, wherein the hTERT has telomerase catalytic activity.

26. The method of claim 24, wherein the hTERT polypeptide has a sequence of  
SEQ ID NO:2.

20

27. The method of claim 24, wherein the cell is from a mammal.

28. The method of claim 27, wherein the cell is human.

25 29. The method of claim 28, wherein the cell is from a human patient.

30. The method of claim 28 or 29, wherein the cell is cultured *in vitro*.

31. The method of claim 29, further comprising the step of reintroducing the cell  
into a human patient.

32. The method of claim 24, wherein the recombinant polynucleotide is introduced into at least one cell in a patient using a gene therapy vector.

33. The method of claim 24, further comprising introducing into the cell a polynucleotide comprising a sequence encoding human telomerase RNA, wherein the polynucleotide is operably linked to a promoter.

34. A method for increasing the proliferative capacity of a vertebrate cell, said method comprising introducing into the cell an effective amount of a human telomerase reverse transcriptase (hTERT) polypeptide.

35. The method of claim 34, wherein the hTERT polypeptide has telomerase catalytic activity.

36. A pharmacological composition comprising a pharmaceutically acceptable carrier and a molecule selected from: a hTERT polypeptide, a polynucleotide encoding a hTERT polypeptide, and a hTERT nucleic acid or subsequence thereof.

37. A method for treatment of a condition associated with an elevated level of telomerase activity within a cell, comprising introducing into said cell a therapeutically effective amount of an inhibitor of said telomerase activity, wherein said inhibitor is an hTERT polypeptide or a hTERT polynucleotide.

38. The method of claim 37, wherein the inhibitor is a polypeptide comprising the sequence of SEQ ID NO:2 or 4, or a subsequence thereof.

39. The method of claim 38, wherein the polypeptide comprises nonstandard or derivatized residues.

40. The method of claim 38, wherein the polypeptide inhibits binding of

endogenous hTERT to hTR.

41. The method of claim 37, wherein the step of introducing the inhibitor into the cell comprises transforming the cell or an ancestor with a polynucleotide encoding the hTERT polypeptide or a hTERT polynucleotide.

42. A vaccine comprising a hTERT polypeptide and an adjuvant.

43. Substantially pure human telomerase comprising human telomerase reverse transcriptase (hTERT) and comprising human telomerase RNA (hTR).

44. The human telomerase of claim 43 that is at least about 95% homogeneous.

45. The human telomerase of claim 43 that is from a cell.

Figure 1

Motif 0

human	AKFLHWLMSVYVVELLSFFYVTETTFQXNR
tez1	ISEIEWLVLGKRSNAKMCLSDFEKRXQIFAEFIYWLXNSFIIPILQSFFYITSSDLRNR
EST2	LKDFRWLFISD---IWFTKHNFFENLNQLAICFISWLPRLIPKIIQTFFYCTEISSTVT-
p123	TREISWHQVET-SAXHPYYFDHEN-IYVLWKLLRWIFEDLVVSLIRCFFYVTEQQKSYSX

. . . . .

Motif 1

human	LFFYRKSVMWSKLQSIGIRQHLKRVQLRDVSEAZVRQHREARPALLTSRLRFIPKP--DGL
tez1	TVYFRKDIWKLRCRPF-I-TSMKMEAFKINENNVRMDTQK-TTLPPAVIRLLPKK--RTP
EST2	IVYFRHDTWNLITPFIIVEYFKTYLVENWVCRNHNSTLS--RPNHSHKRIIPKKSNNEF
p123	TYYYRKNIDVIMKHSI-ADLKKETLAEVQSEVSEWKKK-LGFAPGKLRLIPKK--TTF

. . . . .

Motif 2

human	RPIVNM DYVVGARTFRREKRAERLTSRVKALP-SVLNYERA
tez1	RLITN-LRKRFLIKMGSNKKMLVSTNQTLRPVASILKHLIN EESSGIPFNLEVYMKLLTF
EST2	RIIAIPCRGADEEEFTIYKENHKWAIQPTQKILEYLRNKRPTSFTKIYSPTQIADRIKEF
p123	RPIMTFNKKIVNSDRKTTKLTNTKLLNSHLMLKTLKN-RMFKDPPGPAVFHYDDVMKKY

. . . . .

Motif 3 (A)

tez1	KKDLLKXRMFOR-KKYFVRIDIKSCYDRIKQDLHFRIVKK-KLKDPEPVIRKYATIHATS
EST2	XQRLLKKFNHVLPELYFMKFDVKSCYDSIPRMECHRLKD-ALKNENGFFVRSQYFFNTH
p123	EEFVCKWKQVGPXLPFATHDIEKCYDSVNREKLSTFLKTTXLLSSDFWIMTAQILKRY

. . . . .

Downloaded from www.jstor.org

Figure 2

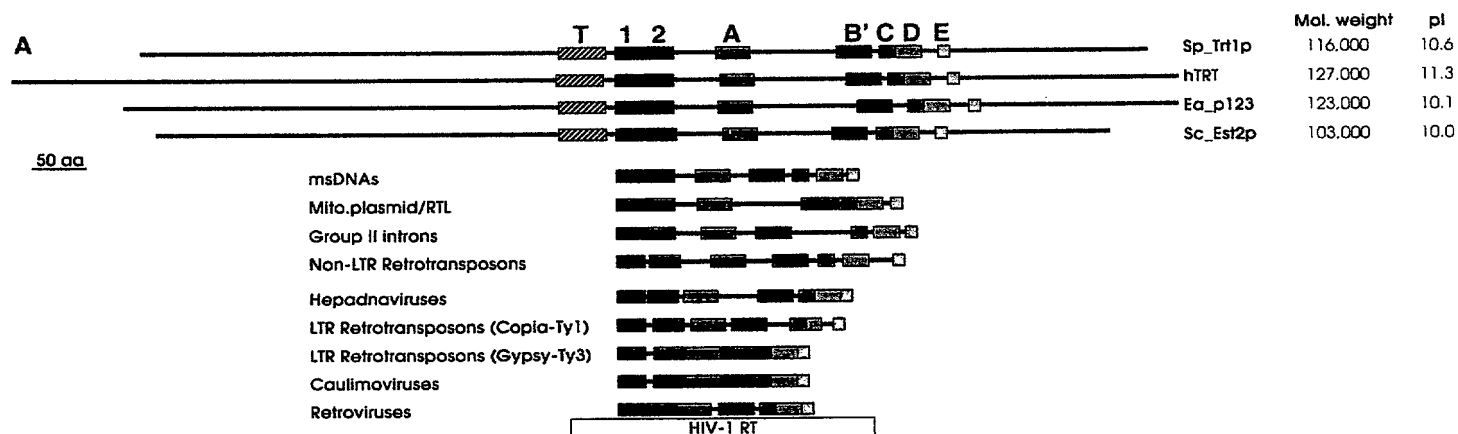


Figure 3

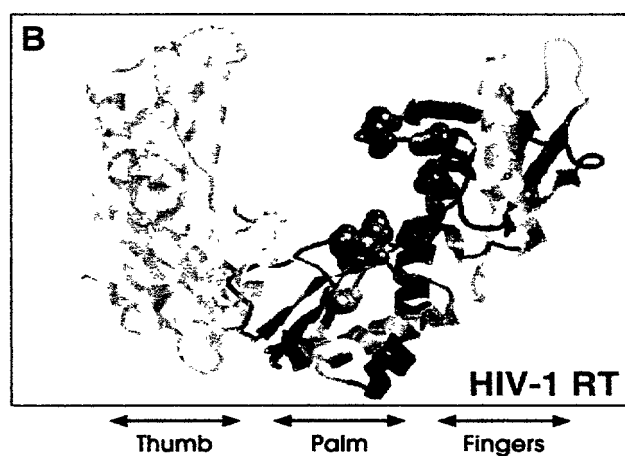




Figure 4

C

Motif T		
TRT con	WL	hh hh pFFY TE p p Y RK W L h I K
Sp_Trt1p	429	WLYNSFIIPILQSFYITESSDLNRRTVYFRKDIWKLLCRPFITSMKM 8
hTRT	546	WLMSVYVVELLRSEFFVTEETTFQKNRLFYRKSVWSKLQSIGIROHLK 10
Ea_p123	441	WIFEDLVVSLIRCFYVTEQQKSYSTKYRKNINWDVIMKMSIADLKK 8
Sc_Est2p	366	WLFRQLIPKIIQTFYCTEISSTVT-IVYFRHDTWNKLITPFIVEYFK 8

Motif 1			Motif 2			Motif A		
TRT con	h	hrhIPKK p	FRhI	h	h	PcLYFh	hdh	CYD I
Sp_Trt1p	NNVRMDTQKTTLP	PAVIRLLPKKNT-	0	FLITNLKRRLIKMGSNKKMLVSTNQTL	40	FGRKKYFVRIDIKSCYDRIKQDLMFRIVKKKLKD	82	
hTRT	EVQRHREARPAL	TSRLRFIPKPDG-	0	LRPIVNMDYVVGARTFRREKRAERLTSRV	45	PPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKP	87	
Ea_p123	KEVEEWKSLGFA	PGKRLRIPKKT-	0	FRPIMTFNKKIVNSDRKTTTLTNTTKLLN	41	GQPKLFFATMDIEKCYDSVNREKLSTFLKTTKLL	100	
Sc_Est2p	CRNHNSYTLN	SFNFHSMRIIPKKSNN	1	FRITAIPCRGADEEEFTIYKENHKNAIQP	42	VLPELYFMKFDVKSCYDSIPRMECMRILKDALKN	68	

Motif B'			Motif C			Motif D		
TRT con	K Y Q	GIPQGS LS hL h Y DL F	LLRL	DDFLhIT	A F	h G c p N cK	W G S	
Sp_Trt1p	SOVLQKVGIPQGS	SILSSFLCHFYMEDLIDEVLSFI	6	LLRVVDDFLFITVNKKD	0	AKKFLNLSLRGFPEKHNFSTSLKTVI	17	KKRMPFF FSV 181
hTRT	KSYVQCQGIPOGS	SILSLCSCYGD MENKLFAGI	5	LLRLVDDFLVTPHLTH	0	AKTFRLTLVRGVPEYGCVVNLKRTVV	19	HGLFPWC LLL 197
Ea_p123	KFYKQTKGIPQGS	LCVSSILSSFYATLEESSLGFL	14	LMRLTDDYLLITTQENN	0	AVLFIEKLINVSRENGFKFMKKLQT	23	QDYCDWI ISI 179
Sc_Est2p	KCYIREDGLFQGS	SLSAPIVDLVYDDLLEFYSEFK	8	ILKLADDFLIISTDQQQ	0	VINIKKLAMGGFQKYNANRDKILA	20	KELEVWKHSST 146

Motif B'			Motif C			Motif D		
TRT con	hPQG	pP hh h	h Y	DDhhh	Gh h	cK h	hLG h	
Sp_Trt1p	TYHKPMLGLPQGS	LISPLCNIVMTLVDNWLEDYI	55	YVRVADDILIGVLGSKN	2	KMIKRDLNNFLNS-LGLTMNEEKTLI	4	ETPARFL YNI
hTRT	RAGQIGAGVPQGS	NLGPILYSIFSSDMPLPHIYHP	7	LSTYADDITVLSSDILA	6	NENYLKTFSDWADKWGISVNAAKTGH	25	ESKQSYL VIL
Ea_p123	GIRYQYNVLPQGS	WKGPSAIFQSSMTKILEPFFKQN	4	IYQYMDLDLYVGSDEIG	1	HRTKIEELRQHLRLWGLTTPDKKHQK	0	EPPFLWM ITL

Item	Value	Unit
1. Total weight of the sample	100.00	g
2. Weight of the sample after drying	95.00	g
3. Weight of the sample after ashing	90.00	g
4. Weight of the sample after extraction	85.00	g
5. Weight of the sample after filtration	80.00	g
6. Weight of the sample after evaporation	75.00	g
7. Weight of the sample after drying	70.00	g
8. Weight of the sample after ashing	65.00	g
9. Weight of the sample after extraction	60.00	g
10. Weight of the sample after filtration	55.00	g
11. Weight of the sample after evaporation	50.00	g
12. Weight of the sample after drying	45.00	g
13. Weight of the sample after ashing	40.00	g
14. Weight of the sample after extraction	35.00	g
15. Weight of the sample after filtration	30.00	g
16. Weight of the sample after evaporation	25.00	g
17. Weight of the sample after drying	20.00	g
18. Weight of the sample after ashing	15.00	g
19. Weight of the sample after extraction	10.00	g
20. Weight of the sample after filtration	5.00	g

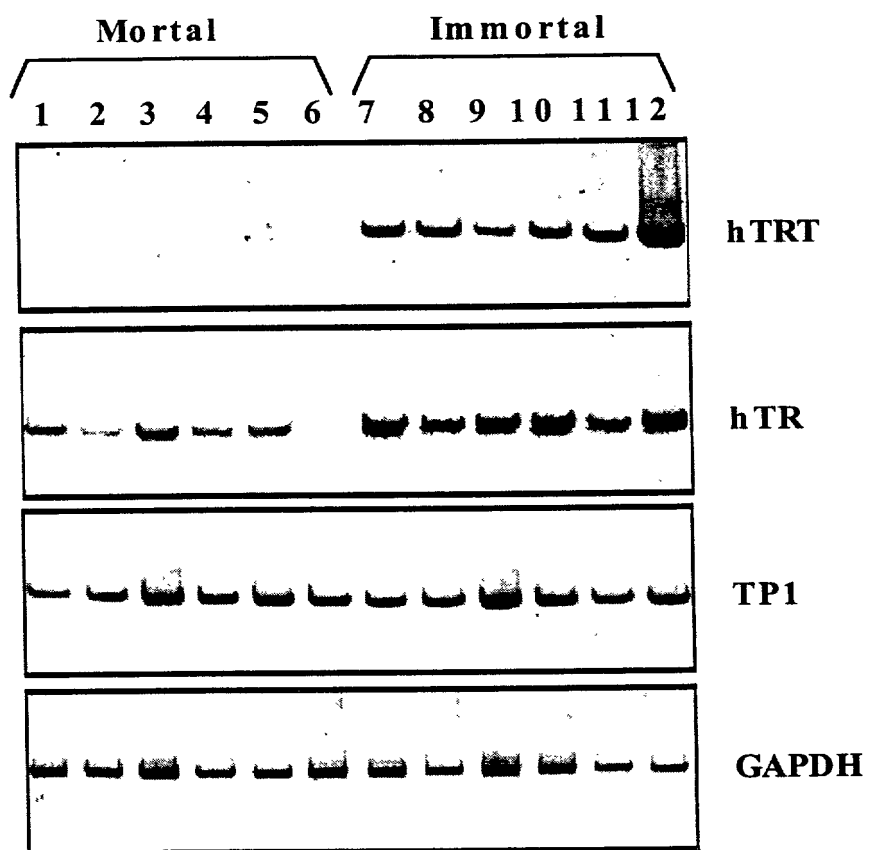


Figure 6

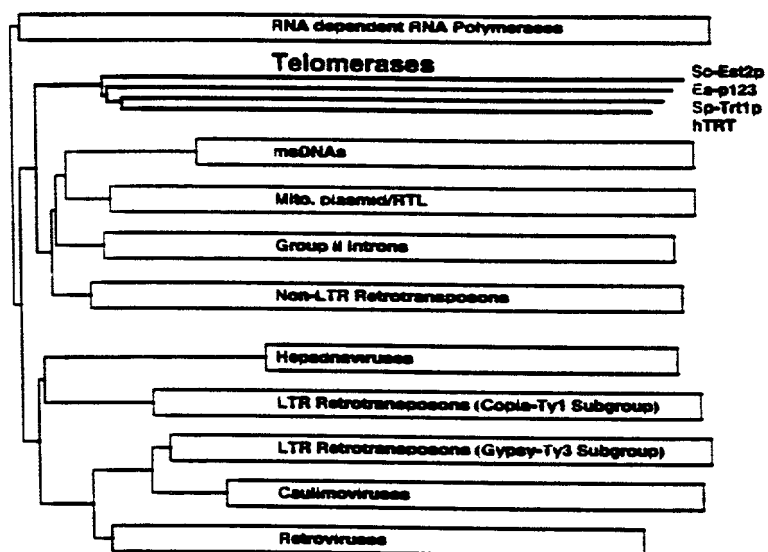


Figure 7

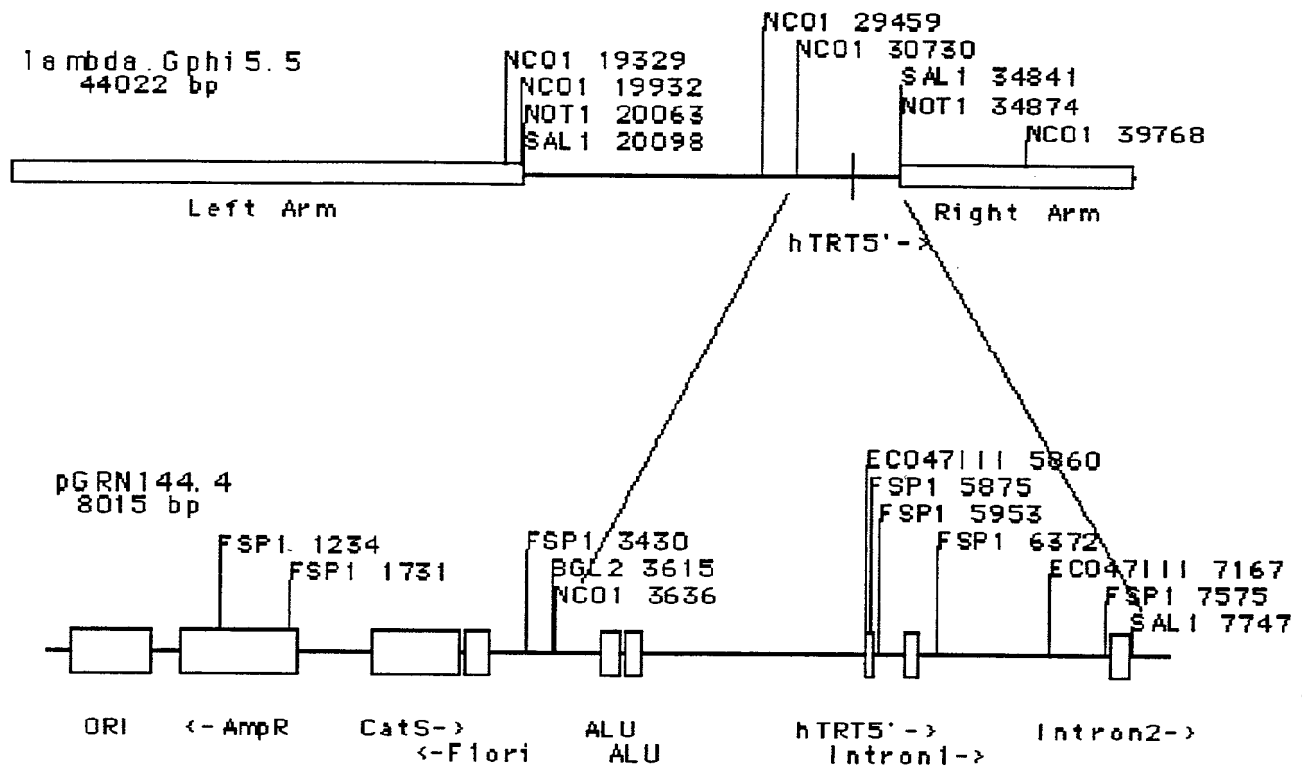


Figure 8

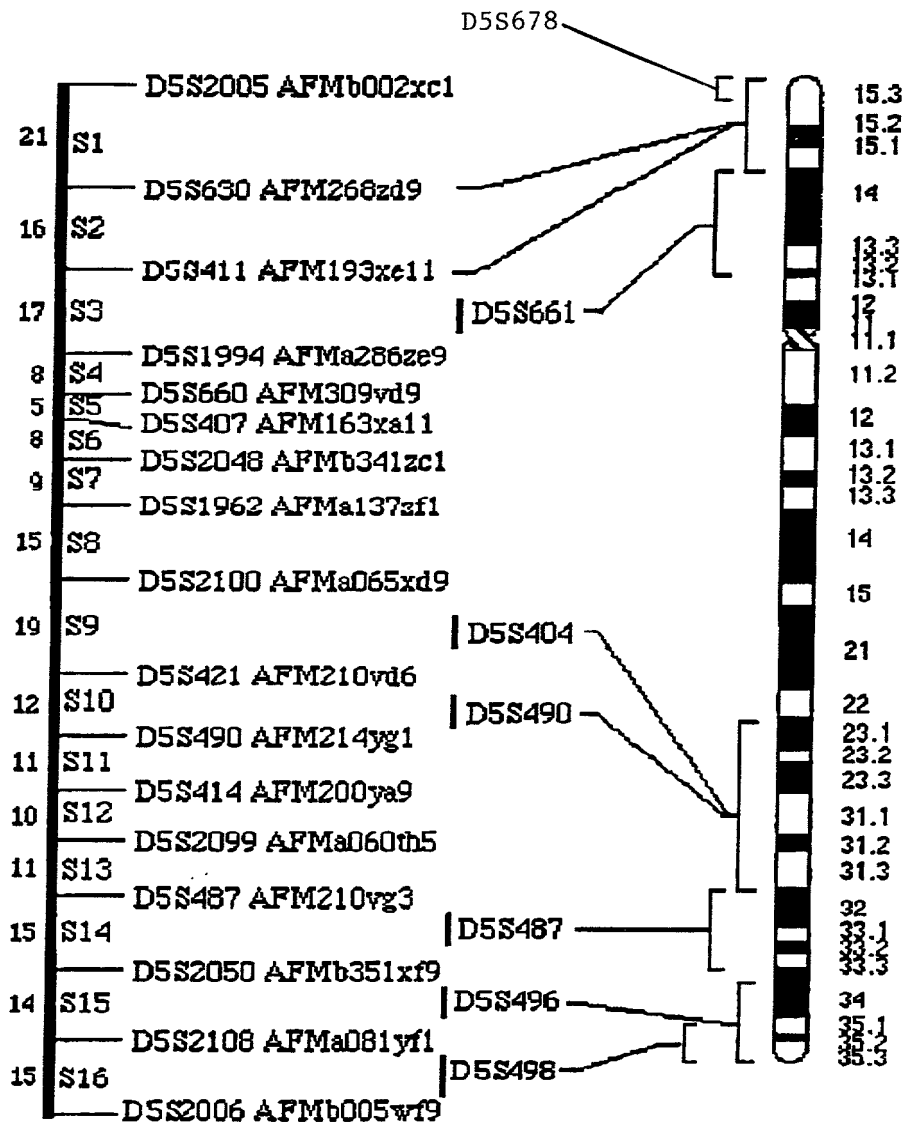


FIGURE 9

# Promoter Reporter Construct

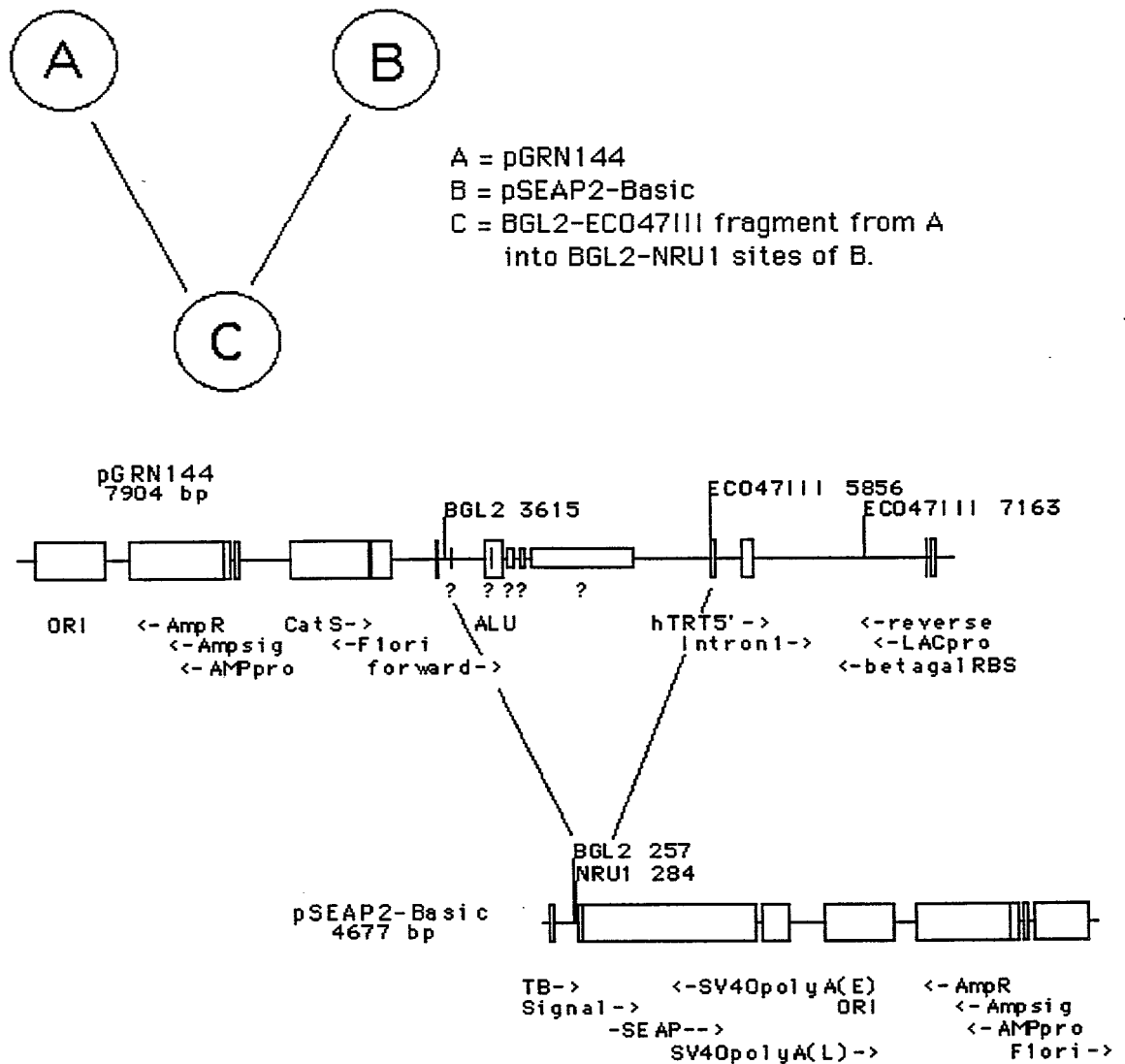


Figure 10  
Page 1

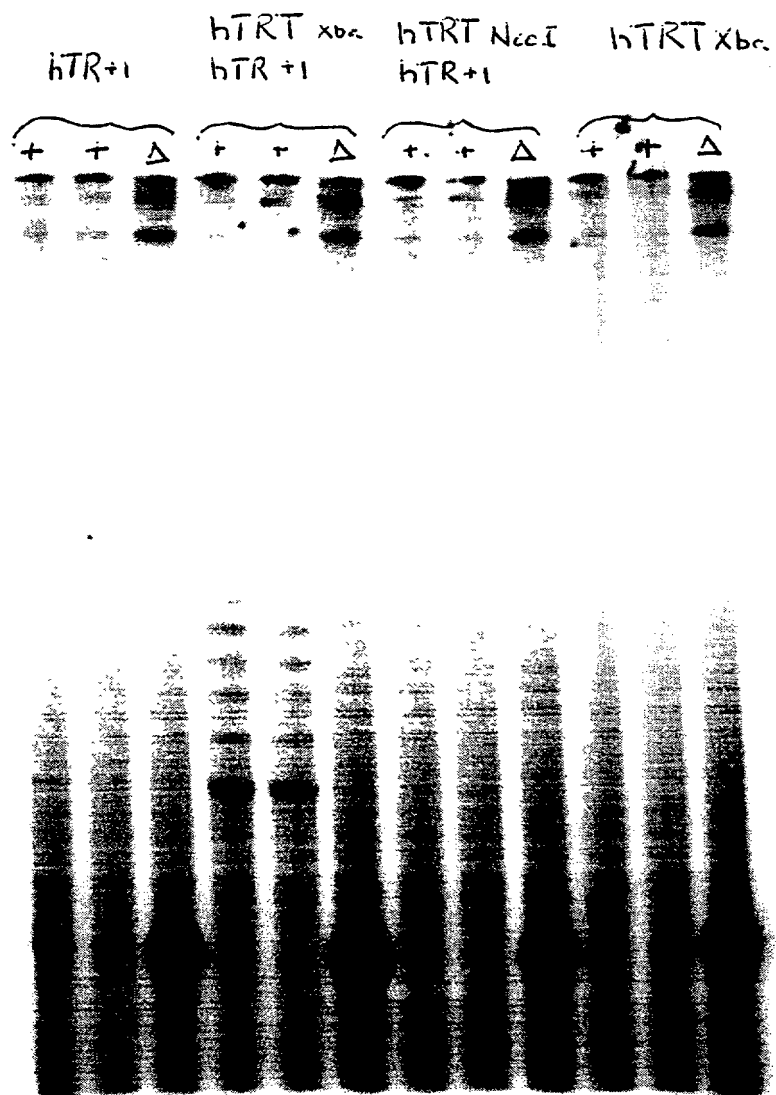
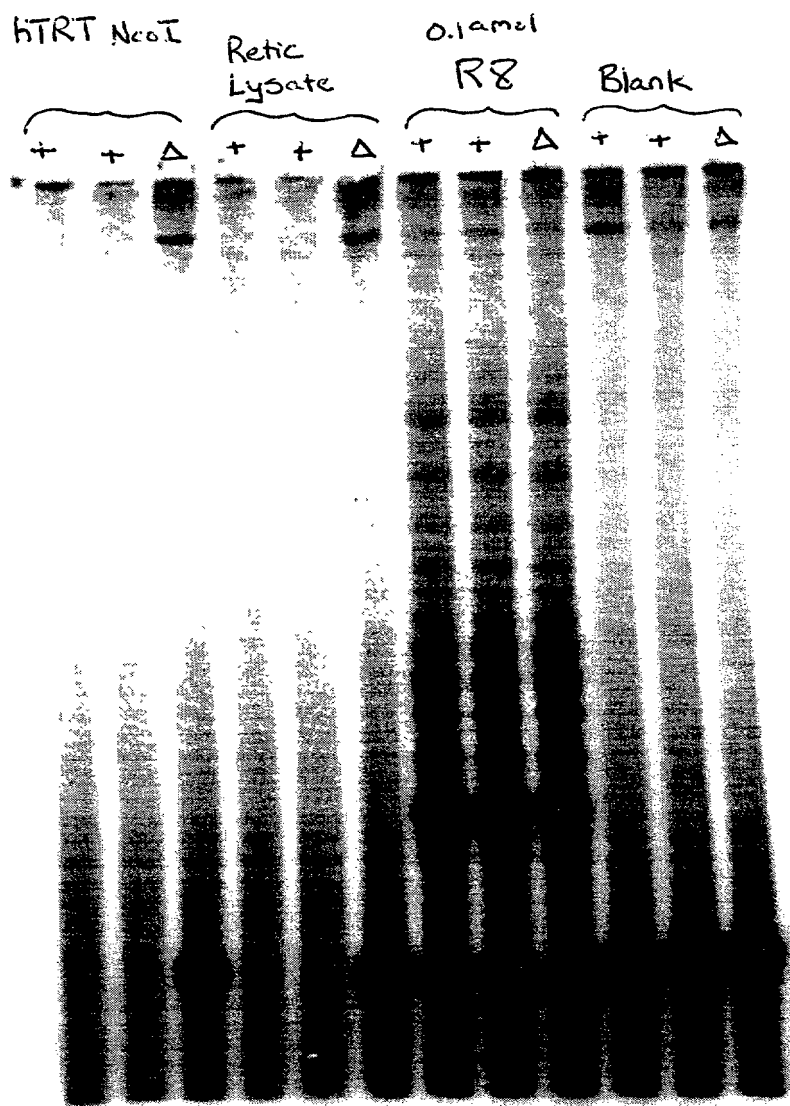


Figure 10  
Page 2



08012951 081497  
264T80" Y56T680



## Telomerase Specific Motifs

	Wl	MOTIF T		MOTIF T'
		FFY TE	Y Rk W l I	E V
TRT con	546	WLMSVYVVELLSFFYVTE	TTFQKNRLEFFYRKSVWSKLQSIGI	13 EAEVR
hTRT	429	WLYNSFIIPILQSFYITE	SSDLNRRTVYFRKDIWKLICRPFI	12 ENNV
spTRT	441	WIFEDLVVSLIRCFYVTE	QQKSYSKTYYYRKNIDVIMKMSI	12 EKEVE
Ea_p123	366	WLFRQLIPKIIQTFYCYTE	ISSTVT.IVYFRHDTWNKLITPFI	9 ENNV
Sc Est2				

# Telomerase RT Motifs (Fingers)

	MOTIF 1	MOTIF 2	MOTIF A	MOTIF B'
	R iPKk	fr I	p lyF D cYD i	Y q GiPQGs lS l Y
TRT con				
hTRT	11 SRLRFIPKPDG 0 LRPIV		69 PELYFVKVDVTGAYDTI 104 YVQCQGI PQGSILSTLLCSLCY	
spTRT	10 AVIRLLPKKNT 0 FRLIT		66 RKKYFVRIDIKSCYDRI 99 YLQKVGIPQGSILSSFLCHFYM	
Ea_p123	10 GKRLRLIPKKTT 0 FRPIM		67 PKLFFATMDIEKCYDSV 117 YKQTKGIPQGLCVSSILSSFYY	
Sc_Est2	13 SKMRIIPKKSN 2 FRIIA		68 PELYFMKFDVKSCYDSI 85 YIREDGLFQGSLSAPIVDLVY	
RT con	p hh h K	hR h	h hDh AF h	hPQG pP hh h
				GY

FIGURE 11

Page 2

# Telomerase RT Motifs (Palm, Primer Grip)

	MOTIF C	MOTIF D	MOTIF E	
TRT con	l l l r l D d f l i t	g n K	w g s l	
hTRT	15 L L L R L V D D F L L V T	15 G V P E Y G C V V N L R K T V V	24 W C G L L L D T R T L	192
spTRT	16 V L L R V V D D F L F I T	15 G F E K H N F S T S L E K T V I	22 F F G F S V N M R S L	176
Ea_p123	24 L L M R L T D D Y L L I T	15 V S R E N G F K F N M K K L Q T	28 W I G I S I D M K T L	174
Sc_Est2	18 L I L K L A D D F L I I S	15 G F Q K Y N A K A N R D K I L A	25 W K H S S T M N N F H	141
RT con	h Y D D h h h	G h h c k h	h L G h	
	F			

FIGURE 12

181 GGACCCGGCGGCTTTCCGCGCGCTGGTGGCCCAGTGCCTGGTGTGCGTGCCCTGGGACGC  
CCTGGGCGCGCCGAAAGGCGCGCGACCACCGGGTCACGGACCACACGCACGGGACCCTGCG

NFkB\_CS1  
GGGRQTYYYQC  
NFkB-MHC-I.2  
TGGGCTTCCCC  
\*\*\*\*\*

241 ACGGCCGCCCCCGCCGCCCCCTCCTTCCGCCAGGTGGGCCTCCCCGGGGTCGGCGTCCG  
TGCCGGCGGGGGGCGGCGGGGGAGGAAGGCGGTCCACCCGGAGGGGCCCCAGCCGCAGGC

Intron1

\*\*\*\*\*

301 GCTGGGGTTGAGGGCGGCCGGGGGGAACCAGCGACATGCGGAGAGCAGCGCAGGCGACTC  
CGACCCCAACTCCCGCCGGCCCCCCTTGGTCGCTGTACGCCTCTCGTCGCGTCCGCTGAG

NFkB\_CS1  
GGGRQTYYYQC  
NFkB\_CS2  
RGGGRMTYYCC  
Topo\_II\_cleavage\_site  
RNYNNCNGYNGKTNYNY  
\*\*\*\*\*>

361 AGGGCGCTTCCCCCGCAGGTGTCCTGCCTGAAGGAGCTGGTGGCCCCAGTGCTGCAGAGG  
TCCCGCGAAGGGGGCGTCCACAGGACGGACTTCCTCGACCACCGGGCTCACGACGTCTCC

254TBD" T52T680

# Figure 13

Page 1

1 AAAACCCCAA AACCCCAAAA CCCCTTTT TAG AGCCCTGCAG TTGGAAATAT  
51 AACCTCAGTA TTAATAAGCT CAGATTTTAA ATATTAATTA CAAAACCTAA  
101 ATGGAGGTTG ATGTTGATAA TCAAGCTGAT AATCATGGCA TTCACTCAGC  
151 TCTTAAGACT TGTGAAGAAA TTAAAGAAGC TAAAACGTTG TACTCTTGGA  
201 TCCAGAAAGT TATTAGATGA AGAAATCAAT CTCAAAGTCA TTATAAAGAT  
251 TTAGAAGATA TTAATAATTT TGCGCAGACA AATATTGTTG CTACTCCACG  
301 AGACTATAAT GAAGAAGATT TTAAAGTTAT TGCAAGAAAA GAAGTATTTT  
351 CAACTGGACT AATGATCGAA CTTATTGACA AATGCTTAGT TGAACCTCTT  
401 TCATCAAGCG ATGTTTCAGA TAGACAAAAA CTTCAATGAT TTGGATTTC  
451 ACTTAAGGGA AATCAATTAG CAAAGACCCA TTTATTAACA GCTCTTTCAA  
501 CTCAAAGCA GTATTTCTTT CAAGACGAAT GGAACCAAGT TAGAGCAATG  
551 ATTGGAAATG AGCTCTTCCG ACATCTCTAC ACTAAATATT TAATATTCCA  
601 GCGAACTTCT GAAGGAACTC TTGTTCAATT TTGCGGGAAT AACGTTTTTG  
651 ATCATTTGAA AGTCAACGAT AAGTTTGACA AAAAGCAAAA AGGTGGAGCA  
701 GCAGACATGA ATGAACCTCG ATGTTGATCA ACCTGCAAAT ACAATGTCAA  
751 GAATGAGAAA GATCACTTTC TCAACAACAT CAACGTGCCG AATTGGAATA  
801 ATATGAAATC AAGAACCAGA ATATTTTATT GCACTCATTT TAATAGAAAT  
851 AACCAATTCT TCAAAAAGCA TGAGTTTGTG AGTAACAAAA ACAATATTTT  
901 AGCGATGGAC AGAGCTCAGA CGATATTCAC GAATATATTC AGATTTAATA  
951 GAATTAGAAA GAAGCTAAAA GATAAGGTTA TCGAAAAAAT TGCCTACATG  
1001 CTTGAGAAAG TCAAAGATTT TAACTTCAAC TACTATTTAA CAAAATCTTG  
1051 TCCTCTTCCA GAAAATTGGC GGAACGGAA ACAAAAAATC GAAAACCTGA  
1101 TAAATAAAAC TAGAGAAGAA AAGTCGAAGT ACTATGAAGA GCTGTTTAGC  
1151 TACACAACCTG ATAATAAATG CGTCACACAA TTTATTAATG AATTTTTCTA  
1201 CAATATACTC CCCAAAGACT TTTTGACTGG AAGAAACCGT AAGAATTTTC  
1251 AAAAGAAAGT TAAGAAATAT GTGGAACATA ACAAGCATGA ACTCATTAC  
1301 AAAAATTAT TGCTTGAGAA GATCAATACA AGAGAAATAT CATGGATGCA  
1351 GGTGAGACC TCTGCAAAGC ATTTTATTA TTTTGATCAC GAAAACATCT  
1401 ACGTCTTATG GAAATTGCTC CGATGGATAT TCGAGGATCT CGTCGTCTCG  
1451 CTGATTAGAT GATTTTTCTA TGTACCCGAG CAACAGAAAA GTTACTCCAA  
1501 AACCTATTAC TACAGAAAGA ATATTTGGGA CGTCATTATG AAAATGTCAA  
1551 TCGCAGACTT AAAGAAGGAA ACGCTTGCTG AGGTCCAAGA AAAAGAGGTT  
1601 GAAGAATGGA AAAAGTCGCT TGGATTGCA CCTGGAAAAC TCAGACTAAT  
1651 ACCGAAGAAA ACTACTTTCC GTCCAATTAT GACTTTCAAT AAGAAGATTG  
1701 TAAATTCAGA CCGGAAGACT ACAAATTAAT CTACAAATAC GAAGTTATTG  
1751 AACTCTCACT TAATGCTTAA GACATTGAAG AATAGAATGT TTAAGATCC  
1801 TTTTGGATTG GCTGTTTTTA ACTATGATGA TGTAATGAAA AAGTATGAGG  
1851 AGTTTGTGTTG CAAATGGAAG CAAGTTGGAC AACCAAAACT CTTCTTTGCA  
1901 ACTATGGATA TCGAAAAGTG ATATGATAGT GTAAACAGAG AAAAATATC  
1951 AACATTCTTA AAAACTACTA AATTACTTTC TTCAGATTTT TGGATTATGA  
2001 CTGCACAAAT TCTAAAGAGA AAGAATAACA TAGTTATCGA TTCGAAAAAC  
2051 TTTAGAAAGA AAGAAATGAA AGATTATTTT AGACAGAAAT TCCAGAAGAT  
2101 TGCATTGAA GGAGGACAAT ATCCAACCTT ATTCAGTGTT CTTGAAAATG  
2151 AACAAAATGA CTTAAATGCA AAGAAAACAT TAATTGTTGA AGCAAAGCAA  
2201 AGAAATTATT TTAAGAAAGA TAACTTACTT CAACCAGTCA TTAATATTTG  
2251 CCAATATAAT TACATTAAT TTAATGGGAA GTTTTATAAA CAAACAAAAG  
2301 GAATTCCTCA AGGTCTTTGA GTTTCATCAA TTTTGTATC ATTTTATTAT

252760

# Figure 13

Page 2

2351 GCAACATTAG AGGAAAGCTC CTTAGGATTC CTTAGAGATG AATCAATGAA  
2401 CCCTGAAAAT CCAAATGTTA ATCTTCTAAT GAGACTTACA GATGACTATC  
2451 TTTTGATTAC AACTCAAGAG AATAATGCAG TATTGTTTAT TGAGAAACTT  
2501 ATAAACGTAA GTCGTGAAAA TGGATTTAAA TTCAATATGA AGAAACTACA  
2551 GACTAGTTTT CCATTAAGTC CAAGCAAATT TGCAAAATAC GGAATGGATA  
2601 GTGTTGAGGA GCAAAATATT GTTCAAGATT ACTGCGATTG GATTGGCATC  
2651 TCAATTGATA TGAAAACCTCT TGCTTTAATG CCAAATATTA ACTTGAGAAT  
2701 AGAAGGAATT CTGTGTACAC TCAATCTAAA CATGCAAACA AAGAAAGCAT  
2751 CAATGTGGCT CAAGAAGAAA CTAAAGTCGT TTTTAATGAA TAACATTACC  
2801 CATTATTTTA GAAAGACGAT TACAACCGAA GACTTTGCGA ATAAAACCTCT  
2851 CAACAAGTTA TTTATATCAG GCGGTTACAA ATACATGCAA TGAGCCAAAG  
2901 AATACAAGGA CCACTTTAAG AAGAACTTAG CTATGAGCAG TATGATCGAC  
2951 TTAGAGGTAT CTAAAATTAT ATACTCTGTA ACCAGAGCAT TCTTTAAATA  
3001 CCTTGTGTGC AATATTAAGG ATACAATTTT TGGAGAGGAG CATTATCCAG  
3051 ACTTTTTCCT TAGCACACTG AAGCACTTTA TTGAAATATT CAGCACAAAA  
3101 AAGTACATTT TCAACAGAGT TTGCATGATC CTCAAGGCAA AAGAAGCAAA  
3151 GCTAAAAAGT GACCAATGTC AATCTCTAAT TCAATATGAT GCATAGTCGA  
3201 CTATTCTAAC TTATTTTGGG AAGTTAATTT TCAATTTTGT TCTTATATAC  
3251 TGGGGTTTTG GGGTTTTGGG GTTTTGGGG



# Figure 15

Page 1

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1 ggtaccgatttactttctttcttcataagctaattgcttcctcgaacgctcctaaatcctctggaaatatttttacaaga 80
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161 ccaagtataaggacaaaaagaacaacttccttccccctaaagacttttactttattaatttacttttcaaatatatttcg 240
241 ggttcgcttacttttaacgtggtactgttttagctgctacttctagccaaccgcgtgtttctaccccgctcattggatat 320
321 agctccttggagtagctcacagaaatccttacaaatccttctgatgagactatattagattcattacagtcctgcatattc 400
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881 actattttatttaaaacgttatgatcagtaggacacttttgcataatatatagttatgcttaatgggttacttgtaacttgc 958

959 ATG ACC GAA CAC CAT ACC CCC AAA AGC AGG ATT CTT CGC TTT CTA GAG AAT CAA TAT GTA 1018
1 M T E H H T P K S R I L R F L E N Q Y V 20

1019 TAC CTA TGT ACC TTA AAT GAT TAT GTA CAA CTT GTT TTG AGA GGG TCG CCG GCA AGC TCG 1078
21 Y L C T L N D Y V Q L V L R G S P A S S 40

1079 TAT AGC AAT ATA TGC GAA CGC TTG AGA AGC GAT GTA CAA ACG TCC TTT TCT ATT TTT CTT 1138
41 Y S N I C E R L R S D V Q T S F S I F L 60

1139 CAT TCG ACT GTA GTC GGC TTC GAC AGT AAG CCA GAT GAA GGT GTT CAA TTT TCT TCT CCA 1198
61 H S T V V G F D S K P D E G V Q F S S P 80

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254T30"K56T680

# Figure 15

Page 2

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81 K C S Q S E 86

1273 CTA ATA GCG AAT GTT GTA AAA CAG ATG TTC GAT GAA AGT TTT GAG CGT CGA AGG AAT CTA 1332  
87 L I A N V V K Q M F D E S F E R R R N L 106

1333 CTG ATG AAA GGG TTT TCC ATG gtaagggtatttctaattgtgaaatattttacctgcaattactgtttcaaagaga 1405  
107 L M K G F S M 113

1406 ttgtattttaaccgataaag AAT CAT GAA GAT TTT CGA GCC ATG CAT GTA AAC GGA GTA CAA AAT 1469  
114 N H E D F R A M H V N G V Q N 128

1470 GAT CTC GTT TCT ACT TTT CCT AAT TAC CTT ATA TCT ATA CTT GAG TCA AAA AAT TGG CAA 1529  
129 D L V S T F P N Y L I S I L E S K N W Q 148

1530 CTT TTG TTA GAA AT gtaaataccgggttaagatgttgcgacatttgaacaagactgacaagtatag T ATC GGC 1601  
149 L L L E I I G 155

1602 AGT GAT GCC ATG CAT TAC TTA TTA TCC AAA GGA AGT ATT TTT GAG GCT CTT CCA AAT GAC 1661  
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1662 AAT TAC CTT CAG ATT TCT GGC ATA CCA CTT TTT AAA AAT AAT GTG TTT GAG GAA ACT GTG 1721  
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1722 TCA AAA AAA AGA AAG CGA ACC ATT GAA ACA TCC ATT ACT CAA AAT AAA AGC GCC CGC AAA 1781  
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1782 GAA GTT TCC TGG AAT AGC ATT TCA ATT AGT AGG TTT AGC ATT TTT TAC AGG TCA TCC TAT 1841  
216 E V S W N S I S I S R F S I F Y R S S Y 235

1842 AAG AAG TTT AAG CAA G gtaactaataactgttatcccttcataactaatttttag AT CTA TAT TTT AAC  
1907

236 K K F K Q D L Y F N 245

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1968 CAA TTT GGA CTT ATA AAC GCA TTT CAA GTG AAG CAA TTG CAC AAA GTG ATT CCA CTG GTA 2027  
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2028 TCA CAG AGT ACA GTT GTG CCC AAA CGT CTC CTA AAG GTA TAC CCT TTA ATT GAA CAA ACA 2087  
286 S Q S T V V P K R L L K V Y P L I E Q T 305

2088 GCA AAG CGA CTC CAT CGT ATT TCT CTA TCA AAA GTT TAC AAC CAT TAT TGC CCA TAT ATT 2147  
306 A K R L H R I S L S K V Y N H Y C P Y I 325

2148 GAC ACC CAC GAT GAT GAA AAA ATC CTT AGT TAT TCC TTA AAG CCG AAC CAG GTG TTT GCG 2207  
326 D T H D D E K I L S Y S L K P N Q V F A 345

2208 TTT CTT CGA TCC ATT CTT GTT CGA GTG TTT CCT AAA TTA ATC TGG GGT AAC CAA AGG ATA 2267  
346 F L R S I L V R V F P K L I W G N Q R I 365

2268 TTT GAG ATA ATA TTA AAA G gtattgtataaaaatttattaccactaaccgattttaccag AC CTC GAA ACT 2336  
366 F E I I L K D L E T 375



# Figure 15

Page 3

2337	TTC TTG AAA TTA TCG AGA TAC GAG TCT TTT AGT TTA CAT TAT TTA ATG AGT AAC ATA AAG	2396
376	F L K L S R Y E S F S L H Y L M S N I K	395
2397	gtaatatgccaaatttttttaccattaattaacaatcag ATT TCA GAA ATT GAA TGG CTA GTC CTT GGA	2465
396	I S E I E W L V L G	405
2466	AAA AGG TCA AAT GCG AAA ATG TGC TTA AGT GAT TTT GAG AAA CGC AAG CAA ATA TTT GCG	2525
406	K R S N A K M C L S D F E K R K Q I F A	425
2526	GAA TTC ATC TAC TGG CTA TAC AAT TCG TTT ATA ATA CCT ATT TTA CAA TCT TTT TTT TAT	2585
426	E F I Y W L Y N S F I I P I L Q S F F Y	445
2586	ATC ACT GAA TCA AGT GAT TTA CGA AAT CGA ACT GTT TAT TTT AGA AAA GAT ATT TGG AAA	2645
446	I T E S S D L R N R T V Y F R K D I W K	465
2646	CTC TTG TGC CGA CCC TTT ATT ACA TCA ATG AAA ATG GAA GCG TTT GAA AAA-ATA AAC GAG	2705
466	L L C R P F I T S M K M E A F E K I N E	485
2706	gtatttttaagatattttttgcataaagctaatattttcag AAC AAT GTT AGG ATG GAT ACT CAG AAA ACT	2775
486	N N V R M D T Q K T	495
2776	ACT TTG CCT CCA GCA GTT ATT CGT CTA TTA CCT AAG AAG AAT ACC TTT CGT CTC ATT ACG	2835
496	T L P P A V I R L L P K K N T F R L I T	515
2836	AAT TTA AGA AAA AGA TTC TTA ATA AAG gtattaatttttggatcatcaatgtactttactttctaattctatta	2906
516	N L R K R F L I K	524
2907	ttagcag ATG GGT TCA AAC AAA AAA ATG TTA GTC AGT ACG AAC CAA ACT TTA CGA CCT GTG	2967
525	M G S N K K M L V S T N Q T L R P V	542
2968	GCA TCG ATA CTG AAA CAT TTA ATC AAT GAA GAA AGT AGT GGT ATT CCA TTT AAC TTG GAG	3027
543	A S I L K H L I N E E S S G I P F N L E	562
3028	GTT TAC ATG AAG CTT CTT ACT TTT AAG AAG GAT CTT CTT AAG CAC CGA ATG TTT GG gtaat	3088
563	V Y M K L L T F K K D L L K H R M F G	581
3089	tatataatgcgcgattccctcattattaattttgcag G CGT AAG AAG TAT TTT GTA CGG ATA GAT ATA	3155
582	R K K Y F V R I D I	591
3156	AAA TCC TGT TAT GAT CGA ATA AAG CAA GAT TTG ATG TTT CGG ATT GTT AAA AAG AAA CTC	3215
592	K S C Y D R I K Q D L M F R I V K K K L	611
3216	AAG GAT CCC GAA TTT GTA ATT CGA AAG TAT GCA ACC ATA CAT GCA ACA AGT GAC CGA GCT	3275
612	K D P E F V I R K Y A T I H A T S D R A	631
3276	ACA AAA AAC TTT GTT AGT GAG GCG TTT TCC TAT T gtaagttttttttttcattggaatttttttaacaa	3343
632	T K N F V S E A F S Y F	643
3344	atttttttttag TT GAT ATG GTG CCT TTT GAA AAA GTC GTG CAG TTA CTT TCT ATG AAA ACA	3405
644	D M V P F E K V V Q L L S M K T	659
3406	TCA GAT ACT TTG TTT GTT GAT TTT GTG GAT TAT TGG ACC AAA AGT TCT TCT GAA ATT TTT	3465
660	S D T L F V D F V D Y W T K S S S E I F	679
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680	K M L K E H L S G H I V K	692

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Page 4

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693																						708							
3594	att	ctg	tca	tct	ttt	ttg	tgt	cat	ttc	tat	atg	gaa	gat	ttg	att	gat	gaa	tac	cta	tcg		3653							
709	i	l	s	s	f	l	c	h	f	y	m	e	d	l	i	d	e	y	l	s		728							
3654	ttt	acg	aaa	aag	aaa	gga	tca	gtg	ttg	tta	cg	gta	gtc	gac	gat	ttc	ctc	ttt	ata	aca		3713							
729	f	t	k	k	k	g	s	v	l	l	r	v	v	d	d	f	l	f	i	t		748							
3714	gtt	aat	aaa	aag	gat	gca	aaa	aaa	ttt	ttg	aat	tta	tct	tta	aga	g	gtg	agt	gtc	gtc	att	ccc	3777						
749	v	n	k	k	d	a	k	k	f	l	n	l	s	l	r	g							764						
3778	ta	agt	tct	ta	acc	gtt	ga	ag	ga	aaa	cac	aat	ttt	tct	acg	agc	ctg	gag	aaa	aca	gta		3840						
765																						778							
3841	ata	aac	ttt	gaa	aat	agt	aat	ggg	ata	ata	aac	aat	act	ttt	ttt	aat	gaa	agc	aag	aaa		3900							
779	i	n	f	e	n	s	n	g	i	i	n	n	t	f	f	n	e	s	k	k		798							
3901	aga	atg	cca	ttc	ttc	ggt	ttc	tct	gtg	aac	atg	agg	tct	ctt	gat	aca	ttg	tta	gca	tgt		3960							
799	r	m	p	f	f	g	f	s	v	n	m	r	s	l	d	t	l	l	a	c		818							
3961	cct	aaa	att	gat	gaa	gcc	tta	ttt	aac	tct	aca	tct	gta	gag	ctg	acg	aaa	cat	atg	ggg		4020							
819	p	k	i	d	e	a	l	f	n	s	t	s	v	e	l	t	k	h	m	g		838							
4021	aaa	tct	ttt	ttt	tac	aaa	att	cta	ag	gt	ata	ct	gt	gta	act	ga	ata	at	ag	ct	gac	aaa	ata	at	cag	a	tcg		4089
839	k	s	f	f	y	k	i	l	r																			848	
4090	agc	ctt	gca	tcc	ttt	gca	caa	gta	ttt	att	gac	att	acc	cac	aat	tca	aaa	ttc	aat	tct		4149							
849	s	l	a	s	f	a	q	v	f	i	d	i	t	h	n	s	k	f	n	s		868							
4150	tgc	tgc	aat	ata	tat	agg	cta	gga	tac	tct	atg	tgt	atg	aga	gca	caa	gca	tac	tta	aaa		4209							
869	c	c	n	i	y	r</																							

## Figure 15

Page 5

4666 gctgacccccaaagcaagcatactataggatttctagtaaagtaaaattaatctcgttattagttttgattgacttgtct 4745  
4746 ttatcctttatacttttaagaaagattgacagtgggtgctgactactgcccacatgcccattaaacgggagtggttaaaca 4825  
4826 ttaaaagtaatacatgaggctaattctcctttcat ttagaataaggaaagtgggttttctataatgaataatgcccgacta 4905  
  
4906 atgcaaaaagacgaagattatcttctaaacaagggggattaaagcatatccgaaggaaaagagagtaatataccagtggt 4985  
4986 gttgaagaaagcaaggataatttggacaagcttctgcagatgacaggctaaattttggcgaccgaattttggtaaaagc 5065  
5066 cccagggttatccatgggtggccggccttgctactgagacgaaaagaaactaaggatagtttgaataactaatagctcattta 5145  
5146 atgtcttatataagggttttggtttttcctgacttcaatttttgcatgggtgaaaagaaatagtggttaagccattattggat 5225  
5226 tccgaaatagccaaatttcttggttcctcaaagcggaaagtctaaagaacttattgaagcttatgaggcttcaaaaactcc 5305  
5306 tcctgattttaaggaggaatcttccaccgatgaggaaatggatagcttatcagctgctgaggagaagcctaattttttgc 5385  
5386 aaaaaagaaaatatcattgggagacatctcttgatgaatcagatgcggagagtatctccagcggatccttgatgtcaata 5465  
5466 acttctattttctgaaatgtatggtcctactgtcgtctcgacttctcgtagctctacgcagttaagtgaccaaaggtacc 5544

4666 4746 4826 4906 4986 5066 5146 5226 5306 5386 5466



**FIGURE 16**  
page 2  
(Seq. ID. No. 1)

2941 cggcttcaag gctgggagga acatgcgtcg caaactcttt ggggtcttgc ggctgaagtg  
3001 tcacagcctg tttctggatt tgcaggtgaa cagcctccag acggtgtgca ccaacatcta  
3061 caagatcctc ctgctgcagg cgtacaggtt tcacgcatgt gtgctgcagc tcccatttca  
3121 tcagcaagtt tggaagaacc ccacattttt cctgcgcgtc atctctgaca cggcctccct  
3181 ctgctactcc atcctgaaag ccaagaacgc agggatgtcg ctgggggcca agggcgccgc  
3241 cggccctctg ccctccgagg ccgtgcagtg gctgtgccac caagcattcc tgetcaagct  
3301 gactcgacac cgtgtcacct acgtgccact cctggggtca ctcaggacag ccagacgca  
3361 gctgagtcgg aagctcccgg ggacgacgct gactgccctg gaggccgcag ccaaccggc  
3421 actgccctca gacttcaaga ccatcctgga ctgatggcca ccgcccaca gccaggccga  
3481 gagcagacac cagcagccct gtcacgccgg gctctacgtc ccagggaggg aggggoggcc  
3541 cacaccaggg ccgcaccgc tgggagtctg aggcctgagt gagtgtttgg ccgaggcctg  
3601 catgtccggc tgaaggctga gtgtccggct gaggcctgag cgagtgtcca gccaagggct  
3661 gagtgtccag cacacctgcc gtcttcactt cccacaggc tggcgctcgg ctccaccca  
3721 gggccagctt ttcctcacca ggagcccggc ttccactccc cacataggaa tagtccatcc  
3781 ccagattcgc cattgttcac ccctcgccct gccctccttt gccttcacc cccaccatcc  
3841 aggtggagac cctgagaagg accctgggag ctctgggaat ttggagtga caaagggtgtg  
3901 ccctgtacac aggcgaggac cctgcacctg gatgggggtc cctgtgggtc aaattggggg  
3961 gaggtgctgt gggagtaaaa tactgaatat atgagttttt cagttttgaa aaaaa

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**FIGURE 17**  
**HUMAN TRT PROTEIN SEQUENCE**  
(SEQ. NO. 2)

MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDP  
AAFRALVAQCLVCVPWDARPPPAAPSFRQVSCLELVARVLQRLCERGAKNVLAFGFA  
LLDGARGGPPEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLARCALFV  
LVAPSCAYQVCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPA  
PGARRRGGASASRLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPA  
RPAAEATSLEGALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVYAETKHFLYS  
SGDKEQLRPSFLLSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPL  
FLELLGNHAQCPYGVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQ  
LLRQHSSPWQVYGFVRACLRLRLVPPGLWGSRHNERFLRNTKKFISLGKHAKLSLQEL  
TWKMSVRDCAWLRRSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLRSFFYVTET  
TFQKNRLFFYRKSVWSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFI  
PKPDGLRPIVNMDYVVGARTFRREKRAERLTSRVKALFVNLNERARRPGLLGASVLG  
LDDIHRAWRTFVLRVRAQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYC  
VRRYAVVQKAAHGHVRKAFKSHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIEQSSSL  
NEASSGLFDVFLRFMCHHAVRIRGKSYVQCQGIQGSILSTLLCSLCYGD MENKLFAG  
IRRDGLLLRLVDDFLLVTPHLTHAKTFLRTLVRGVPEYGCVVNLKRKTVVNF PVEDEAL  
GGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDYSSYARTSIRASLTFNRGFKAGRNMRR  
KLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILLQAYRFHACVLQLPFHQVWKNPT  
FFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLPSEAVQWLCHQAFLCLKLTRHRVT  
YVPLLGSRLTAQTQLSRKLPGTTLTALEAAANPALPSDFKTILD

**FIGURE 18**  
Clone 712562  
(SEQ ID NO. 3)

GGCCAAGTTCCTGCACTGGCTGATGAGTGTGTACGTCGTCGAGCTGCTCAGGTCTTTCTT  
TTATGTCACGGAGACCACGTTTCAAAAGAACAGGCTCTTTTTCTACCGGAAGAGTGTCTG  
GAGCAAGTTGCAAAGCATTGGAATCAGACAGCACTTGAAGAGGGTGCAGCTGCGGGAGCT  
GTCGGAAGCAGAGGTGAGGCAGCATCGGGAAGCCAGGCCCGCCCTGCTGACGTCCAGACT  
CCGCTTCATCCCCAAGCCTGACGGGCTGCGGCCGATTGTGAACATGGACTACGTCGTGGG  
AGCCAGAACGTTCCGCAGAGAAAAGAGGGGCCGAGCGTCTCACCTCGAGGGTGAAGGCACT  
GTTTACGCGTGCTCAACTACGAGCGGGCGCGGCCCGCCCTCCTGGGCGCCTCTGTGCT  
GGGCCTGGACGATATCCACAGGGCCTGGCGCACCTTCGTGCTGCGTGTGCGGGCCCCAGGA  
CCCGCCGCCTGAGCTGTACTTTGTCAAGGTGGATGTGACGGGCGCGTACGACACCATCCC  
CCAGGACAGGCTCACGGAGGTATCGCCAGCATCATCAAACCCAGAACACGTAAGTGCCT  
GCGTCGGTATGCCGTGGTCCAGAAGGCCCGCCATGGGCACGTCCGCAAGGCCCTTCAAGAG  
CCACGTCTACGTCCAGTGCCAGGGGATCCCGCAGGGCTCCATCCTCTCCACGCTGCTCT  
GCAGCCTGTGCTACGGCGACATGGAGAACAAGCTGTTTTCGGGGGATTTCGGCGGGACGGGC  
TGCTCCTGCGTTTGGTGGATGATTTCTTGTGTTGGTGACACCTCACCTCACCCACGCGAAAA  
CCTTCCTCAGGACCCTGGTCCGAGGTGTCCCTGAGTATGGCTGCGTGGTGAAGTTGCGGA  
AGACAGTGGTGAAGTTCCCTGTAGAAGACGAGGCCCTGGGTGGCACGGCTTTTGTTCAGA  
TGCCGGCCCCACGGCCTATTTCCCTGGTGCGGCCTGCTGCTGGATACCCGGACCCTGGAGG  
TGCAGAGCGACTACTCCAGCTATGCCCGGACCTCCATCAGAGCCAGTCTCACCTTCAACC  
GCGGCTTCAAGGCTGGGAGGAACATGCGTCGCAAACCTCTTGGGGTCTTGCGGCTGAAGT  
GTCACAGCCTGTTTCTGGATTTGCAGGTGAACAGCCTCCAGACGGTGTGCACCAACATCT  
ACAAGATCCTCCTGCTGCAGGCGTACAGGTTTACGCGATGTGTGCTGCAGCTCCCATTTT  
ATCAGCAAGTTTGAAGAACCCACATTTTCTGCGCGTCATCTCTGACACGGCCTCCC  
TCTGCTACTCCATCCTGAAAGCCAAGAACGCAGGGATGTGCTGGGGCCAAGGGCGCCG  
CCGGCC7TCTGCCCTCCGAGGCCGTGCAGTGGCTGTGCCACCAAGCATTCTGCTCAAGC  
TGACTCGACACCGTGTACCTACGTGCCACTCCTGGGGTCACTCAGGACAGCCCAGACGC  
AGCTGAGTCGGAAGCTCCCGGGGACGACGCTGACTGCCCTGGAGGCCGAGCCAACCCGG  
CACTGCCCTCAGACTTCAAGACCATCCTGGACTGATGGCCACCCGCCACAGCCAGGCCG  
AGAGCAGACACCAGCAGCCCTGTACGCCGGGCTCTACGTCCAGGGAGGGAGGGGCGGC  
CCACACCCAGGCCTGCACCGCTGGGAGTCTGAGGCCTGAGTGAGTGTTTGGCCGAGGCCT  
GCATGTCCGGCTGAAGGCTGAGTGTCCGGCTGAGGCCTGAGCGAGTGTCCAGCCAAGGGC  
TGAGTGTCCAGCACACCTGCCGTCTTCACTTCCCCACAGGCTGGCGCTCGGCTCCACCCC  
AGGGCCAGCTTTTCTCACCAGGAGCCCGGCTTCCACTCCCCACATAGGAATAGTCCATC  
CCCAGATTGCGCATTTGTTACCCCTCGCCCTGCCCTCCTTTGCCCTTCCACCCCCACCATC  
CAGGTGGAGACCCTGAGAAGGACCCTGGGAGCTCTGGGAATTTGGAGTGACCAAAGGTGT  
GCCCTGTACACAGGCGAGGACCCTGCACCTGGATGGGGTCCCTGTGGGTCAAATTGGGG  
GGAGGTGCTGTGGGAGTAAATACTGAATATATGAGTTTTTTCAGTTTTTG0AAAAAAAAA  
AAAAAAAAAAAAAAAAA





# FIGURE 20

Page 1

## SEQUENCE NO. 4 (DNA) AND SEQUENCE NO. 5 (PROTEIN) (TRANSLATION OF A $\Delta$ 182 hTRT VARIANT)

```

1
met
GCAGCGCTGCGTCCTGCTGCGCACGTGGGAAGCCCTGGCCCCGGCCACCCCCGCG ATG

10
pro arg ala pro arg cys arg ala val arg ser leu leu arg ser
CCG CGC GCT CCC CGC TGC CGA GCC GTG CGC TCC CTG CTG CGC AGC

20
his tyr arg glu val leu pro leu ala thr phe val arg arg leu
CAC TAC CGC GAG GTG CTG CCG CTG GCC ACG TTC GTG CGG CGC CTG

30
gly pro gln gly trp arg leu val gln arg gly asp pro ala ala
GGG CCC CAG GGC TGG CGG CTG GTG CAG CGC GGG GAC CCG GCG GCT

40
phe arg ala leu val ala gln cys leu val cys val pro trp asp
TTC CGC GCG CTG GTG GCC CAG TGC CTG GTG TGC GTG CCC TGG GAC

50
ala arg pro pro pro ala ala pro ser phe arg gln val ser cys
GCA CGG CCG CCC CCC GCC GCC CCC TCC TTC CGC CAG GTG TCC TGC

60
leu lys glu leu val ala arg val leu gln arg leu cys glu arg
CTG AAG GAG CTG GTG GCC CGA GTG CTG CAG AGG CTG TGC GAG CGC

70
gly ala lys asn val leu ala phe gly phe ala leu leu asp gly
GGC GCG AAG AAC GTG CTG GCC TTC GGC TTC GCG CTG CTG GAC GGG

80
ala arg gly gly pro pro glu ala phe thr thr ser val arg ser
GCC CGC GGG GGC CCC CCC GAG GCC TTC ACC ACC AGC GTG CGC AGC

90

```

## Page 2

260 270  
ala his pro gly arg thr arg gly pro ser asp arg gly phe cys  
GCC CAC CCG GGC AGG ACG CGT GGA CCG AGT GAC CGT GGT TTC TGT

# FIGURE 20

Page 3

280  
val val ser pro ala arg pro ala glu glu ala thr ser leu glu  
GTG GTG TCA CCT GCC AGA CCC GCC GAA GAA GCC ACC TCT TTG GAG

290 300  
gly ala leu ser gly thr arg his ser his pro ser val gly arg  
GGT GCG CTC TCT GGC ACG CGC CAC TCC CAC CCA TCC GTG GGC CGC

310  
gln his his ala gly pro pro ser thr ser arg pro pro arg pro  
CAG CAC CAC GCG GGC CCC CCA TCC ACA TCG CGG CCA CCA CGT CCC

320 330  
trp asp thr pro cys pro pro val tyr ala glu thr lys his phe  
TGG GAC ACG CCT TGT CCC CCG GTG TAC GCC GAG ACC AAG CAC TTC

340  
leu tyr ser ser gly asp lys glu gln leu arg pro ser phe leu  
CTC TAC TCC TCA GGC GAC AAG GAG CAG CTG CGG CCC TCC TTC CTA

350 360  
leu ser ser leu arg pro ser leu thr gly ala arg arg leu val  
CTC AGC TCT CTG AGG CCC AGC CTG ACT GGC GCT CGG AGG CTC GTG

370  
glu thr ile phe leu gly ser arg pro trp met pro gly thr pro  
GAG ACC ATC TTT CTG GGT TCC AGG CCC TGG ATG CCA GGG ACT CCC

380 390  
arg arg leu pro arg leu pro gln arg tyr trp gln met arg pro  
CGC AGG TTG CCC CGC CTG CCC CAG CGC TAC TGG CAA ATG CGG CCC

400  
leu phe leu glu leu leu gly asn his ala gln cys pro tyr gly  
CTG TTT CTG GAG CTG CTT GGG AAC CAC GCG CAG TGC CCC TAC GGG

410 420  
val leu leu lys thr his cys pro leu arg ala ala val thr pro  
GTG CTC CTC AAG ACG CAC TGC CCG CTG CGA GCT GCG GTC ACC CCA

430



# FIGURE 20

Page 5

590 600  
 ile gly ile arg gln his leu lys arg val gln leu arg glu leu  
 ATT GGA ATC AGA CAG CAC TTG AAG AGG GTG CAG CTG CGG GAG CTG

610  
 ser glu ala glu val arg gln his arg glu ala arg pro ala leu  
 TCG GAA GCA GAG GTC AGG CAG CAT CGG GAA GCC AGG CCC GCC CTG

620 630  
 leu thr ser arg leu arg phe ile pro lys pro asp gly leu arg  
 CTG ACG TCC AGA CTC CGC TTC ATC CCC AAG CCT GAC GGG CTG CGG

640  
 pro ile val asn met asp tyr val val gly ala arg thr phe arg  
 CCG ATT GTG AAC ATG GAC TAC GTC GTG GGA GCC AGA ACG TTC CGC

650 660  
 arg glu lys arg ala glu arg leu thr ser arg val lys ala leu  
 AGA GAA AAG AGG GCC GAG CGT CTC ACC TCG AGG GTG AAG GCA CTG

670  
 phe ser val leu asn tyr glu arg ala arg arg pro gly leu leu  
 TTC AGC GTG CTC AAC TAC GAG CGG GCG CGG CGC CCC GGC CTC CTG

680 690  
 gly ala ser val leu gly leu asp asp ile his arg ala trp arg  
 GGC GCC TCT GTG CTG GGC CTG GAC GAT ATC CAC AGG GCC TGG CGC

700  
 thr phe val leu arg val arg ala gln asp pro pro pro glu leu  
 ACC TTC GTG CTG CGT GTG CGG GCC CAG GAC CCG CCG CCT GAG CTG

710 720  
 tyr phe val lys val asp val thr gly ala tyr asp thr ile pro  
 TAC TTT GTC AAG GTG GAT GTG ACG GGC GCG TAC GAC ACC ATC CCC

730  
 gln asp arg leu thr glu val ile ala ser ile ile lys pro gln  
 CAG GAC AGG CTC ACG GAG GTC ATC GCC AGC ATC ATC AAA CCC CAG

264T90" T552T580

**FIGURE 20**

Page 6

740 750  
asn thr tyr cys val arg arg tyr ala val val gln lys ala ala  
AAC ACG TAC TGC GTG CGT CGG TAT GCC GTG GTC CAG AAG GCC GCC

760  
his gly his val arg lys ala phe lys ser his val leu arg pro  
CAT GGG CAC GTC CGC AAG GCC TTC AAG AGC CAC GTC CTA CGT CCA

770 780  
val pro gly asp pro ala gly leu his pro leu his ala ala leu  
GTG CCA GGG GAT CCC GCA GGG CTC CAT CCT CTC CAC GCT GCT CTG

790  
gln pro val leu arg arg his gly glu gln ala val cys gly asp  
CAG CCT GTG CTA CGG CGA CAT GGA GAA CAA GCT GTT TGC GGG GAT

800 807  
ser ala gly arg ala ala pro ala phe gly gly OP  
TCG GCG GGA CGG GCT GCT CCT GCG TTT GGT GGA TGA TTTCTTGTTGGT

GACACCTCACCTCACCCACGCGAAACCTTCCTCAGGACCCTGGTCCGAGGTGTCCCTGA  
GTATGGCTGCGTGGTGAACCTTGCGGAAGACAGTGGTGAACCTTCCCTGTAGAAGACGAGGC  
CCTGGGTGGCACGGCTTTTGTTTCAGATGCCGGCCACGGCCTATTTCCCTGGTGCGGCCT  
GCTGCTGGATAACCGGACCCTGGAGGTGCAGAGCGACTACTCCAGCTATGCCCGGACCTC  
CATCAGAGCCAGTCTCACCTTCAACCGCGGCTTCAAGGCTGGGAGGAACATGCGTCGCAA  
ACTCTTTGGGGTCTTGCGGCTGAAGTGTACAGCCTGTTTCTGGATTTGCAGGTGAACAG  
CCTCCAGACGGTGTGCACCAACATCTACAAGATCCTCCTGCTGCAGGCGTACAGGTTTCA  
CGCATGTGTGCTGCAGCTCCCATTTTCATCAGCAAGTTTGGAAGAACCCACATTTTTCCT  
GCGCGTCATCTCTGACACGGCCTCCCTCTGCTACTCCATCCTGAAAGCCAAGAACGCAGG  
GATGTCGCTGGGGGCCAAGGGCGCCGCCGCCCTCTGCCCTCCGAGGCCGTGCAGTGGCT  
GTGCCACCAAGCATTCCTGCTCAAGCTGACTCGACACCGTGTACCTACGTGCCACTCCT

# FIGURE 20

Page 7

GGGGTCACTCAGGACAGCCCAGACGCAGCTGAGTCGGAAGCTCCCGGGGACGACGCTGAC  
TGCCCTGGAGGCCGCAGCCAACCCGGCACTGCCCTCAGACTTCAAGACCATCCTGGACTG  
ATGGCCACCCGCCCACAGCCAGGCCGAGAGCAGACACCAGCAGCCCTGTCACGCCGGGCT  
CTACGTCCCAGGGAGGGAGGGGCGGCCACACCCAGGCCCGCACCGCTGGGAGTCTGAGG  
CCTGAGTGAGTGTTTGGCCGAGGCCTGCATGTCCGGCTGAAGGCTGAGTGTCGGGCTGAG  
GCCTGAGCGAGTGTCAGCCAAGGGCTGAGTGTCAGCACACCTGCCGTCTTCACTTCCC  
CACAGGCTGGCGCTCGGCTCCACCCCAGGGCCAGCTTTTCCTCACCAGGAGCCCGGCTTC  
CACTCCCCACATAGGAATAGTCCATCCCCAGATTGCGCCATTGTTACCCCCTCGCCCTGCC  
CTCCTTTGCCTTCCACCCCCACCATCCAGGTGGAGACCCTGAGAAGGACCCTGGGAGCTC  
TGGAATTTGGAGTGACCAAAGGTGTGCCCTGTACACAGGCGAGGACCCTGCACCTGGAT  
GGGGGTCCCTGTGGGTCAAATTGGGGGGAGGTGCTGTGGGAGTAAAATACTGAATATATG  
AGTTTTTTCAGTTTTTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

464780 "T56T60"

# FIGURE 21

page 1

RESTRICTION SITES and REGIONS Map of DNA pGRN144.4  
(SEQ. ID. NO. 6)

3601 ATCGATTGGGCCCCGAGATCTCGCGCGCGAGGCCTGCCATGGGACCCACTGCAGGGGCAGC  
TAGCTAACCCGGGCTCTAGAGCGCGCGCTCCGGACGGTACCCTGGGTGACGTCCCCGTCG

3615  
BGL2

3636  
NCO1

3661 TGGGANGCTGCAGGCTTCAGGTCCCAGTGGGGTTGCCATCTGCCAGTAGAAACCTGATGT  
ACCTNCGACGTCCGAAGTCCAGGGTACCCCAACGGTAGACGGTCATCTTTGGACTACA

3721 AGAATCAGGGCGCGAGTGTGGACACTGTCCTGAATCTCAATGTCTCAGTGTGTGCTGAAA  
TCTTAGTCCCGCGCTCACACCTGTGACAGGACTTAGAGTTACAGAGTCACACACGACTTT

3781 CATGTAGAAATTAAAGTCCATCCCTCCTACTCTACTGGGATTGAGCCCCCTCCCTATCCC  
GTACATCTTTAATTTAGGTAGGGAGGATGAGATGACCCTAACTCGGGGAAGGGATAGGG

3841 CCCCCAGGGGCAGAGGAGTTCTCTCACTCCTGTGGAGGAAGGAATGATACTTTGTTATT  
GGGGGTCCCCGTCTCCTCAAGGAGAGTGAGGACACCTCCTTCCTTACTATGAAACAATAA

\*\*\*\*\*

3901 TTTCACTGCTGGTACTGAATCCACTGTTTCATTTGTTGGTTTGTGTTTGTGTTTGTGTTGAGA  
AAAGTGACGACCATGACTTAGGTGACAAAGTAAACAACCAAACAAACAAAACAAAACCTCT

\*\*\*\*\*  
3961 AGCGGTTTCACTCTTGTTGCTCAGGCTGGANGGAGTGCAATGGCGCGATCTTGGCTTACT  
TCGCCAAAGTGAGAACAACGAGTCCGACCTNCCTCACGTTACCGCGCTAGAACCGAATGA

ALU

\*\*\*\*\*  
4021 GCAGCCTCTGCCTCCAGGTTCAAGTGATTCTCCTGCTTCCGCCTCCCATTTGGCTGGGA  
CGTCGGAGACGGAGGGTCCAAGTTCACTAAGAGGACGAAGGCGGAGGGTAAACCGACCCCT

\*\*\*\*\*  
4081 TTACAGGCACCCGCCACCATGCCCAGCTAATTTTTTGTATTTTTTAGTANANACNGGGGTG  
AATGTCCGTGGGCGGTGGTACGGGTCGATTAAAAAACATAAAAAATCATNTNTGNCCCCAC

A

=====

4141 GGGGTGGGGTTCACATGTTGGCCAAGCTGGTCTCGAACTTCTGAACTCAGATGATCCANC  
CCCCACCCCAAGTGTACAACCGGTTGACAGAGCTTGAAGACTTGAGTCTACTAGGTNG

LU

=====

4201 TGCCTCTGCCTCCTAAAATTGCTGGGATTACAGGTGTNANCCACCATGCCCCAACTCAAAA



# FIGURE 21

page 2

ACGGAGACGGAGGATTTTAAACGACCCTAATGTCCACANTNGGTGGTACGGGTTGAGTTTT

4261 TTTACTCTGTTTANAAACATCTGGGTCTAAGGTAGGAANCTCACCCCACTCAATTTTTGT  
AAATGAGACAAATNTTTGTAGACCCAGATTCCATCCTTNGAGTGGGGTGAGTTAAAAACA

4321 GGTGTTTTTAAGCCAATNANAAAATTTTTTNAIGTTGTTTNNNNNNNNNNNNNNNNNNNN  
CCACAAAAATTCGGTTANTNTTTTAAAAAANTACAACAAANNNNNNNNNNNNNNNNNNNNN

4381 NNN  
NN

4441 NNN  
NN

4501 NNN  
NN

4561 NNN  
NN

4621 NNN  
NN

4681 NNN  
NN

4741 NNN  
NN

4801 NNN  
NN

4861 NNN  
NN

4921 NNN  
NN

4981 NNN  
NN

5041 NGCCANGRAGGGGGCCAGGTTCCAANTTCCCAACCKTTTTWGGARGGACNGCCCCAGGG  
NCGGTNCYTCCCCCGGTCCAAGGTTNAAGGGTTGGMAAAWCCCTYCCTGNCGGGGGTCCC

5101 GGGGATRAACAGANTNGGGGGKGGTWWGGTTNAKGGTGGGAACNCCTTNGCGCCTGGAG  
CCCCTAYTTGTCTNANCCCCMCCAACCAANTMCCACCCTTGNGGAANCGSCGGACCTC

5161 AACGTGCAAAGAGGAAATGAAGGGCCTGKGTCAAGGAGCCCAAGTNGGCGGGGRAGTTTG  
TTGACGTTTCTCCTTTACTTCCCGGACMCAAGTTCTCGGGTTCANCCGCCCCYTCAAAC

434300-1562680

# FIGURE 21

page 3

5221 CAGGGAGGCACTCCGGGGAGGTCCSGCGTGCCCGTCCAAGGGAGCAATGCGTCCTTCGGG  
GTCCCTCCGTGAGGCCCTCCAGGSCGCACGGGCAGGTTCCCTCGTTACGCAGGAAGCCC

5281 TTCGTCCCCAWGCCGCGTCTACGCGCCTYCCGTCTCCCTTCACGTTCCGGCATTTCGTG  
AAGCAGGGGTWCGGCGCAGATGCGCGGARGGCAGGAGGGGAAGTGCAAGGCCGTAAGCAC

5341 GTGCCCCGAGCCCGACGCCCCGCGTCCGGACCTGGAGGCAGCCCTGGGTCTCCGGATCAG  
CACGGGCCCTCGGGCTGCGGGGCGCAGGCCTGGACCTCCGTGCGGACCCAGAGGCCTAGTC

5401 GCCAGCGGCCAAAGGGTCGCCGCACGCACCTGTTCCAGGGCCTCCACATCATGGCCCCCT  
CGGTGCGCGGTTTCCAGCGGCGTGCGTGGACAAGGGTCCCGGAGGTGTAGTACCGGGGA

5461 CCCTCGGGTTACCCACAGCCTAGGCCGGATTTCGACCTCTCTCCGCTGGGGCCCTCGCCT  
GGGAGCCCAATGGGGTGTGCGATCCGGCCTAAGCTGGAGAGAGGGCGACCCGGGAGCGGA

## Sp1

\*\*\*\*\*

5521 GGCGTCCCTGCACCCTGGGAGCGCGAGCGGCGCGGGCGGGGAAGCGCGGCCCATACCC  
CCGCAGGGACGTGGGACCCTCGCGCTCGCCGCGCGCCCGCCCCCTTCGCGCCGGGTATGGG

5581 CCGGGTCCGCCCGGAAGCAGCTGCGCTGTGCGGGCCAGGCCGGGCTCCAGTGGATTTCG  
GGCCCAGGCGGGCCTTCGTGACGCGACAGCCCCGGTCCGGCCCCGAGGGTCACCTAAGCG

## Topo\_II\_cleavage\_site

\*\*\*\*\*

5641 GGGCACAGACGCCCAGGACCGCGCTTCCACGTGGCGGAAGGACTGGGGACCCGGGCACC  
CCCGTGTCTGCGGGTCCTGGCGCGAAGGGTGCACCGCCTTCTGACCCCTGGGCCCCGTGG

## E2F

\*\*\*\*\*

5701 CGTCCTGCCCCCTTCACCTTCCAGCTCCGCTTCTTCGCGCGGACCCGGCCCCGTCCCGAA  
GCAGGACGGGGAAAGTGGAAGGTCGAGGCGAAGAAGGCGCGCCTGGGCCGGGGCAGGGCTT

## E

\*\*\*\*

5761 CCCTTCCAGGTCCCGGCCAGCCCCCTTCGGGGCCCTCCAGCCCCCTCCCCTTCTTTTC  
GGGAAGGGTCCAGGGCCGGGTGCGGGGAAGGCCCGGGAGGGTCGGGGAGGGGAAGGAAAAG

49480" T562T680

# FIGURE 21

page 4

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                Sp1
            =====
2F              NFkB              h
*****          *****
5821 CGCGGCCCCGCCCTCTCCTTCGCGGGCGCGAGTTTCAGGCAGCGCTGCGTCCTGCTGCGCA
    GCGCCGGGGCGGGAGAGGAAGCGCCGCGCTCAAAGTCCGTCGCGACGCAGGACGACGCGT
                                ^               ^
                                5860           5875
                                ECO47III      FSP1

TRT5'
*****>
5881 CGTGGGAAGCCCTGGCCCCGGCCACCCCCGCGATGCCGCGCGCTCCCCGCTGCCGAGCCG
    GCACCCCTTCGGGACCGGGGCGGTGGGGGCGCTACGGCGCGCGAGGGGCGACGGCTCGGC

5941 TGCCTCCCTGCTGCGCAGCCACTACCGCGAGGTGCTGCCGCTGGCCACGTTTCGTGCGGC
    ACGCGAGGGACGACGCGTGGTGATGGCGCTCCACGACGGCGACCGGTGCAAGCACGCCG
        ^
        5953
        FSP1

6001 GCCTGGGGCCCCAGGGCTGGCGGCTGGTGACGCGGGGACCCGGCGGCTTTCCGCGCGC
    CGGACCCCGGGTCCCGACCGCCGACACGTCGCGCCCTGGGCGCGCGAAAGGCGCGC

6061 TGGTGGCCCAGTGCCCTGGTGTGCGTGCCCTGGGACGCACGGCCGCCCCCGCGCCCCCT
    ACCACCGGGTCACGGACCACACGCACGGGACCCTGCGTGCCGGCGGGGGCGCGGGGGA

                NFkB
            =====

                *****
6121 CCTTCGCCAGGTGGGCCTCCCCGGGGTCCGCGTCCGCTGGGGTTGAGGGCGGCCGGGG
    GGAAGGCGGTCCACCCGAGGGGCCCCAGCCGACGGCCGACCCCAACTCCCGCCGGCCCC

                                Topo_II_cleavage_s
                                ::::::::::::::
                                NFkB
                                =====

Intron1
*****>
6181 GGAACCAGCGACATGCGGAGAGCAGCGCAGGCGACTCAGGGCGCTTCCCCCGCAGGTGTC
    CCTTGGTCGCTGTACGCCTCTCGTCGCGTCCGCTGAGTCCCGCAAGGGGGCGTCCACAG

ite
:
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# FIGURE 21

page 5

6241 CTGCCTGAAGGAGCTGGTGGCCCGAGTGCTGCAGAGGCTGTGCGAGCGCGGCGGAAGAA  
GACGGACTTCCTCGACCACCGGGCTCACGACGTCTCCGACACGCTCGCGCCGCGCTTCTT

6301 CGTGCTGGCCTTCGGCTTCGCGCTGCTGGACGGGGCCCGCGGGGGCCCCCGAGGCCTT  
GCACGACCGGAAGCCGAAGCGCGACGACCTGCCCGGGCGCCCCCGGGGGGGCTCCGGAA

6361 CACCACCAGCGTGCAGCTACCTGCCCAACACGGTGACCGACGCACTGCGGGGGAGCGG  
GTGGTGGTTCGACGCGTCGATGGACGGGTGTGCCACTGGCTGCGTGACGCCCCCTCGCC  
^  
6372  
FSP1

6421 GGCCTGGGGGCTGCTGCTGCGCCGCGTGGGCGACGACGTGCTGGTTACCTGCTGGCACG  
CCGACCCCCGACGACGCGGGCGACCCGCTGCTGCACGACCAAGTGGACGACCGTGC

6481 CTGCGCGCTCTTTGTGCTGGTGGCTCCAGCTGCGCCTACCAGGTGTGCGGGCCGCCGCT  
GACGCGCGAGAAACACGACCACCGAGGGTCGACGCGGATGGTCCACACGCCCGGCGGCGA

6541 GTACCAGCTCGGGCTGCCACTCAGGCCCGGGCCCCGCCACACGCTAGTGGACCCCGAAG  
CATGGTCGAGCCGCGACGGTGAGTCCGGGCCGGGGCGGTGTGCGATCACCTGGGGCTTC

6601 GCGTCTGGGATGCGAACGGGCCTGGAACCATAGCGTCAGGGAGGCCGGGGTCCCCCTGGG  
CGCAGACCCTACGCTTGCCCGACCTTGGTATCGCAGTCCCTCCGGCCCCAGGGGGACCC

6661 CCTGCCAGCCCCGGGTGCGAGGAGGCGCGGGGGCAGTGCCAGCCGAAGTCTGCCGTTGCC  
GGACGGTCGGGGCCCACGCTCCTCCGCGCCCCCGTCACGGTCGGCTTCAGACGGCAACGG

6721 CAAGAGGCCCAGGCGTGGCGCTGCCCCTGAGCCGGAGCGGACGCCCGTTGGGCAGGGGTC  
GTTCTCCGGGTCCGCACCGCGACGGGGACTCGGCCTCGCCTGCGGGCAACCCGTCCCCAG

6781 CTGGGCCCACCGGGCAGGACGCGTGGACCGAGTGACCGTGGTTTCTGTGTGGTGTACC  
GACCCGGGTGGGCCCGTCTGCGCACCTGGCTCACTGGCACCAAAGACACACCACAGTGG

6841 TGCCAGACCCGCCGAAGAAGCCACCTCTTTGGAGGGTGCGCTCTCTGGCACGCGCCACTC  
ACGGTCTGGGCGGCTTCTTCGGTGGAGAAACCTCCACGCGAGAGACCGTGC GCGGTGAG

6901 CCACCCATCCGTGGGCGGCCAGCACACGCGGGCCCCCATCCACATCGCGGCCACCACG  
GGTGGGTAGGCACCCGGCGGTGCTGGTGC GCGCCGGGGGGTAGGTGTAGCGCCGGTGGTGC

6961 TCCCTGGGACACGCCTTGTCCTCCCGGTGTACGCCGAGACCAAGCACTTCCTCTACTCCTC  
AGGGACCCTGTGCGGAACAGGGGGCCACATGCGGCTCTGGTTTCGTGAAGGAGATGAGGAG

7021 AGGCGACAAGGAGCAGCTGCGGCCCTCCTTCCTACTCAGCTCTCTGAGGCCAGCCTGAC  
TCCGCTGTTCTCTGTCGACGCCGGGAGGAAGGATGAGTCGAGAGACTCCGGGTGCGACTG

7081 TGGCGCTCGGAGGCTCGTGGAGACCATCTTTCTGGGTTCCAGGCCCTGGATGCCAGGGAC  
ACCGCGAGCCTCCGAGACCTCTGGTAGAAAGACCCAAGGTCCGGGACCTACGGTCCCTG

0091251.03149

# FIGURE 21

page 6

7141 TCCCCGCAGGTTGCCCCGCTGCCCCAGCGCTACTGGCAAATGCGGCCCTGTTTCTGGA  
AGGGGCGTCCAACGGGGCGGACGGGGTTCGCGATGACCGTTTACGCCGGGGACAAAGACCT

7167

ECO47III

7201 GCTGCTTGGAACCACGCGCAGTGCCCCCTACGGGGTGCTCCTCAAGACGCACTGCCCCGCT  
CGACGAACCCTTGGTGCGCTCACGGGGATGCCCCACGAGGAGTTCTGCGTGACGGGGCGA

7261 GCGAGCTGCGGTACCCCCAGCAGCCGGTGTCTGTGCCCCGGGAGAAGCCCCAGGGCTCTGT  
CGCTCGACGCCAGTGGGGTCGTTCGGCCACAGACACGGGCCCTCTTCGGGGTCCCCGAGACA

7321 GCGGGCCCCGAGGAGGAGGACACAGACCCCCGTCGCCTGGTGAGCTGCTCCGCCAGCA  
CCGCCGGGGGCTCCTCCTCCTGTGTCTGGGGGCAGCGGACCACGTCGACGAGGCGGTCTGT

7381 CAGCAGCCCCCTGGCAGGTGTACGGCTTCGTGCGGGCCTGCCTGCGCCGGCTGGTGCCCCC  
GTCGTTCGGGGACCGTCCACATGCCGAAGCACGCCCGGACGGACGCGGCCGACCACGGGGG

7441 AGGCCTCTGGGGCTCCAGGCACAACGAACGCCGCTTCCTCAGGAACACCAAGAAGTTCAT  
TCCGGAGACCCCGAGGTCCGTGTTGCTTGCGGCGAAGGAGTCCTTGTGGTTCTTCAAGTA

7501 CTCCCTGGGGAAGCATGCCAAGCTCTCGCTGCAGGAGCTGACGTGGAAGATGAGCGTGCG  
GAGGGACCCCTTCGTACGGTTCGAGAGCGACGTCCTCGACTGCACCTTCTACTCGCACGC

\*\*\*\*\*

7561 GGACTGCGCTTGGCTGCGCAGGAGCCCAGGTGAGGAGGTGGTGGCCGTCGAGGGCCCAGG  
CCTGACGCGAACCAGCGCTCCTCGGGTCCACTCCTCCACCACCGGCAGCTCCCGGGTCC

7575

FSP1

Intron2

7621 CCCAGAGCTGAATGCAGTAGGGGCTCAGAAAAGGGGGCAGGCAGAGCCCTGGTCCTCCT  
GGGGTCTCGACTTACGTCATCCCCGAGTCTTTCCCCCGTCCGTCTCGGGACCAGGAGGA

7681 GTCTCCATCGTACGTGGGCACACGTGGCTTTTCGCTCAGGACGTCGAGTGGACACGGTG  
CAGAGGTAGCAGTGACCCGTGTGCACCGAAAAGCGAGTCCTGCAGCTCACCTGTGCCAC

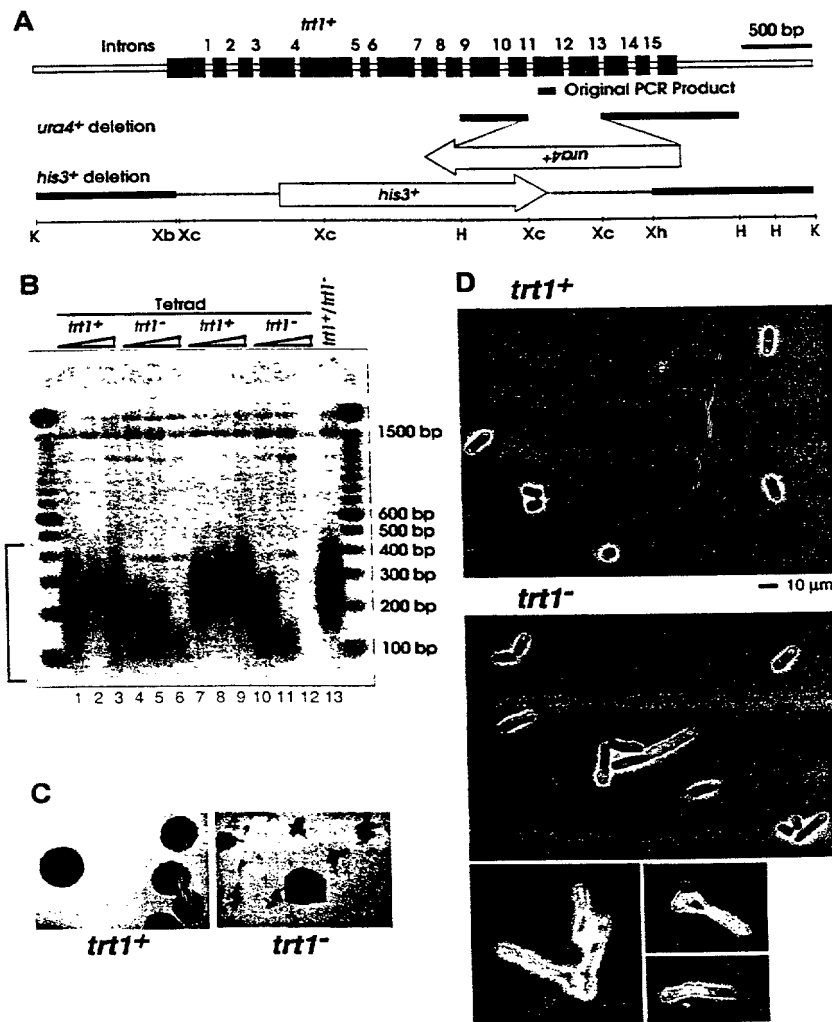
\*\*>

7741 ATCGAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCATA  
TAGCTCCAGCTGAGATCTCCTAGGGGCCCATGGCTCGAGCTTAAGCATTAGTACCAGTAT

7747

SAL1

Figure 22



**FIGURE 23**  
EST AA281296  
(Seq. ID. No. 8)

gc  
caagttcctg cactggctga tgagtgtgta cgtcgtcgag ctgctcaggt ctttctttta  
tgtcacggag accacgtttc aaaagaacag gctctttttc taccggaaga gtgtctggag  
caagttgcaa agcattggaa tcagacagca cttgaagagg gtgcagctgc gggacgtgtc  
ggaagcagag gtcaggcagc atcggaagc caggcccgcc ctgctgacgt ccagactccg  
cttcatcccc aagcctgacg ggctgcggcc gattgtgaac atggactacg tcgtgggagc  
cagaacgttc cgcagagaaa agagggccga gcgtctcacc tcgaggggtga aggcactgtt  
cagcgtgctc aactacgagc gggcgcg

031296.034437

FIGURE 24  
(Seq. ID. No. 9)

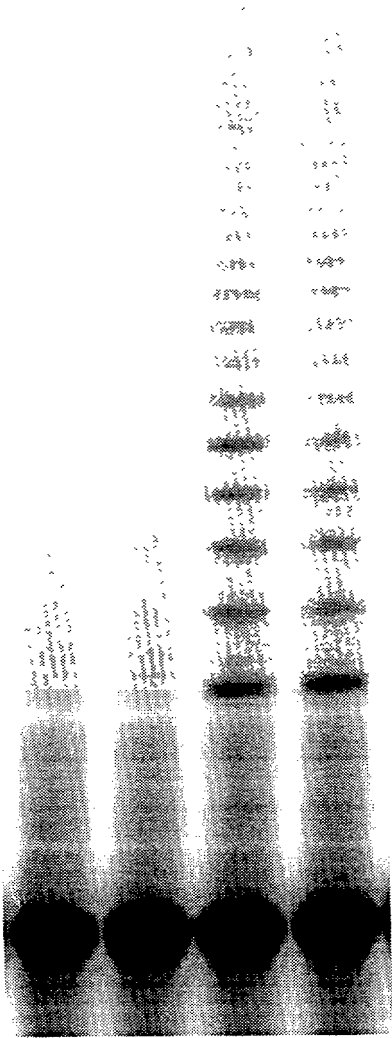
TCTACCTTGACAGACCTCCAGCCGTACATGCGACAGTTCGTGGCTCACCTGCAGGAG  
ACCAGCCCGCTGAGGGATGCCGTCGTCATCGAGCAGAGCTCCTCCCTGAATGAGGC  
CAGCAGTGGCCTCTTCGACGTCTTCCTACGCTTCATGTGCCACCACGCCGTGCGCAT  
CAGGGGCAAGTC

64730 752750



Figure 25

pBBS212 pGRN133



← INTERNAL CONTROL

approximate cell #    5,000   5,000   5,000   5,000

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **HUMAN TELOMERASE CATALYTIC SUBUNIT: DIAGNOSTIC AND THERAPEUTIC METHODS**

the specification of which:

  X   is attached hereto or  
       was filed on            as Application No.            and was amended on            (if applicable).

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56. I claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign applications(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

**Prior Foreign Application(s)**

Country	Application No.	Date of Filing	Priority Claimed Under 35 USC 119
			Yes <u>  </u> No <u>  </u>
			Yes <u>  </u> No <u>  </u>

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date

I claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Date of Filing	Status
08/854,050	May 9, 1997	<u>  </u> Patented <u>  X  </u> Pending <u>  </u> Abandoned
08/851,843	May 6, 1997	<u>  </u> Patented <u>  X  </u> Pending <u>  </u> Abandoned
08/846,017	April 25, 1997	<u>  </u> Patented <u>  X  </u> Pending <u>  </u> Abandoned
08/844,419	April 18, 1997	<u>  </u> Patented <u>  X  </u> Pending <u>  </u> Abandoned
08/724,643	October 1, 1996	<u>  </u> Patented <u>  X  </u> Pending <u>  </u> Abandoned

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

**William M. Smith, Reg. No. 30,223**

**Randolph T. Apple, Reg. No. 36,429**

**Joe Liebeschuetz, Reg. No. 37,505**

**Greg P. Einhorn, Reg. No. 38,440**

Send Correspondence to: <b>William M. Smith</b> <b>TOWNSEND and TOWNSEND and CREW LLP</b> <b>Two Embarcadero Center, 8th Floor</b> <b>San Francisco, CA 94111-3834</b>	Direct Telephone Calls to: (Name, Reg. No., Telephone No.)  <b>Name: William M. Smith</b> <b>Reg. No.: 30,223</b> <b>Telephone: (415) 576-0200</b>
--	---

Full Name of Inventor 1	Last Name <b>Cech</b>	First Name <b>Thomas</b>	Middle Name or Initial <b>R.</b>	
Residence & Citizenship	City <b>Boulder</b>	State/Foreign Country <b>Colorado</b>	Country of Citizenship <b>United States</b>	
Post Office Address	Post Office Address <b>1545 Rockmont Circle</b>	City <b>Boulder</b>	State/Country <b>Colorado</b>	Zip Code <b>80303</b>
Full Name of Inventor 2	Last Name <b>Lingner</b>	First Name <b>Joachim</b>	Middle Name or Initial	
Residence & Citizenship	City <b>Boulder</b>	State/Foreign Country <b>Colorado</b>	Country of Citizenship <b>Switzerland</b>	
Post Office Address	Post Office Address <b>360 30th Street</b>	City <b>Boulder</b>	State/Country <b>Colorado</b>	Zip Code <b>80303</b>
Full Name of Inventor 3	Last Name <b>Nakamura</b>	First Name <b>Toru</b>	Middle Name or Initial	
Residence & Citizenship	City <b>Boulder</b>	State/Foreign Country <b>Colorado</b>	Country of Citizenship <b>Japan</b>	
Post Office Address	Post Office Address <b>4940 Thunderbird Circle #204</b>	City <b>Boulder</b>	State/Country <b>Colorado</b>	Zip Code <b>80303</b>
Full Name of Inventor 4	Last Name <b>Chapman</b>	First Name <b>Karen</b>	Middle Name or Initial <b>B.</b>	
Residence & Citizenship	City <b>Sausalito</b>	State/Foreign Country <b>California</b>	Country of Citizenship <b>United States</b>	
Post Office Address	Post Office Address <b>71 Cloud View Road</b>	City <b>Sausalito</b>	State/Country <b>California</b>	Zip Code <b>94965</b>

Full Name of Inventor 5	Last Name <b>Morin</b>	First Name <b>Gregg</b>	Middle Name or Initial <b>B.</b>	
Residence & Citizenship	City <b>Palo Alto</b>	State/Foreign Country <b>California</b>	Country of Citizenship <b>United States</b>	
Post Office Address	Post Office Address <b>3407 Janice Way</b>	City <b>Palo Alto</b>	State/Country <b>California</b>	Zip Code <b>94303</b>
Full Name of Inventor 6	Last Name <b>Harley</b>	First Name <b>Calvin</b>	Middle Name or Initial <b>B.</b>	
Residence & Citizenship	City <b>Palo Alto</b>	State/Foreign Country <b>California</b>	Country of Citizenship <b>Canada</b>	
Post Office Address	Post Office Address <b>1730 University Avenue</b>	City <b>Palo Alto</b>	State/Country <b>California</b>	Zip Code <b>94301</b>
Full Name of Inventor 7	Last Name <b>Andrews</b>	First Name <b>William</b>	Middle Name or Initial	
Residence & Citizenship	City <b>Richmond</b>	State/Foreign Country <b>California</b>	Country of Citizenship <b>United States</b>	
Post Office Address	Post Office Address <b>6102 Park Avenue</b>	City <b>Richmond</b>	State/Country <b>California</b>	Zip Code <b>94805</b>

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 1  <b>Thomas R. Cech</b>	Signature of Inventor 2  <b>Joachim Lingner</b>	Signature of Inventor 3  <b>Toru Nakamura</b>
Date	Date	Date
Signature of Inventor 4  <b>Karen B. Chapman</b>	Signature of Inventor 5  <b>Gregg B. Morin</b>	Signature of Inventor 6  <b>Calvin B. Harley</b>
Date	Date	Date
Signature of Inventor 7  <b>William Andrews</b>		
Date		